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LETTER FROM THE EDITORS

Dr. Elmer L. DeGowin and Dr. Richard V. Ebert have completed six-year terms on the Board of Editors. On behalf of the Central Society for Clinical Research and the Editorial Board, we wish to express our appreciation for their valuable services. An additional vacancy was created by the death of Dr. Frank H. Bethell.

Dr. Harper K. Hellems, Dr. Robert F. Schilling, and Dr. Gene H. Stollerman have accepted appointments, and Dr. Richard L. Landau has accepted reappointment on the Board of Editors.

During the past year the Editors and Board members have called upon many of their associates and colleagues in other universities to aid them in the evaluation of manuscripts. Those who have participated in this capacity in the past year are:

Ackermann, W. Wilbur
Baker, Burton L.
Bernstein, Isadore A.
Berson, Solomon A.
Beutler, Ernest
Bishop, Ronald C.
Block, Walter D.
Bohr, David F.
Bollett, Alfred J.
Braude, Abraham I.
Bricker, Neal S.
Brown, Audrey K.
Burch, George E., Jr.
Carr, Edward A., Jr.
Castor, C. William
Chandler, Joseph P.
Christensen, Halvor N.
Conn, Jerome W.
Conway, F. James
Coon, Minor J.
Copeland, Arthur H.
Corcoran, A. C.
Davenport, Fred M.
Davenport, Horace W.
Davey, Winthrop N.
Freeman, Smith
French, A. James
French, Arthur B.
Gordon, Irving
Gurney, Clifford W.
Hall, Wendell H.
Hanke, Martin
Harrell, E. Richard, Jr.
Heller, Ben I.
Henley, Keith S.
Hennessy, Albert V.
Hoobler, Sibley W.
Jacobson, Leon O.
Johnston, Franklin D.
Kappas, Attallah
Kerby, Grace P.
Koenig, Virgil L.
Korst, Donald R.
Lepper, Mark H.
Linman, James W.
Littel, Arthur S.
Love, William D.
Masouredis, Serafeim
Mathews, Kenneth P.
Maxwell, James H.
Paine, Thomas F.
Payne, Francis E.
Pressman, David
Remington, Richard D.
Reynolds, Ernest W.
Richards, Dickinson W.
Riley, Gardner M.
Roseman, Saul
Ross, Joseph F.
Schilling, Robert F.
Schneider, Richard C.
Sherry, Sol
Shetlar, Marvin R.
Sloan, Herbert E., Jr.
Solari, Arthur J.
Stollerman, Gene H.
Straffon, Ralph A.
Streeten, David H. P.
Sutton, H. Eldon
Tourtellotte, Wallace W.
Tsao, Makepiece U.
Vallee, Bert L.
Vermeulen, Cornelius W.
Vilter, Richard W.
Volwiler, Wade

Denko, Charles W.
Donaldson, Virginia
Dudley, Horace C.
Duff, Ivan F.
Dustan, Harriet P.
Fajans, Stefan S.
Finch, Clement A.
Fletcher, Anthony P.
Freedberg, A. Stone
Meyers, Muriel C.
Mikkelson, William M.
Moloney, William C.
Moore, Carl V.
Neel, James V.
Norcia, Leonard N.
Nungester, Walter J.
Olson, Robert E.
Owen, Charles A.
Wegst, Audrey V.
Weisman, Russell, Jr.
Weller, John M.
Williams-Ashman, H. G.
Wilson, James L.
Witebsky, Ernest
Woods, Lauren A.
Wroblewski, Felix

We wish to take this opportunity to thank the past and present members of the Editorial Board for their excellent services to the JOURNAL and to the Central Society for Clinical Research. We would also like to express our appreciation for the cooperation received from the C. V. Mosby Company.

WILLIAM D. ROBINSON, M.D., EDITOR
WILLIAM H. BEIERWALTES, M.D., ASSOCIATE EDITOR

THE "SOCIALIZATION" OF RESEARCH FUNDS

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FELLOW members of the Central Society and guests: I would like to express my sincere appreciation for the high honor conferred upon me in being elected to the presidency of this distinguished organization. It has been a real privilege to have been allowed to participate in the affairs of the Society as a member of the Board of Editors, as a Councillor, and now as an officer. I have become increasingly aware, during this rich experience, of the remarkable talents of the young men and women who form our membership and of the high quality of the research that they have contributed. I would also like to pay tribute to the faithful diligence, loyalty, and patience of the Council, and to the integrity they bring to the performance of their duties of selecting a program and making recommendations of new members. These are not easy tasks. Special words of gratitude go to our Secretary, Dr. Weisberger, and to his secretary, Miss Lois Reeves, upon whose shoulders falls the tedious, routine business of making our Society function. With people such as these to guide me, my job has been pleasant and easy, and I have learned that the President is not such an important individual as I had once thought.

About a week ago, while driving my car across northern Illinois, I heard a news broadcast carrying the warning of Surgeon General Burney that those in the field of medical sciences in the United States, operating through, or in, cooperation with the federal government, must immediately inaugurate a "crash program" of intensive medical school- and hospital building, with sharp expansion of training programs, in order to keep pace with the demands that lie ahead, which are arising out of the explosive increase in our population.

Almost immediately thereafter, in the magazine, *U. S. News and World Report,* appeared an excellent article on the same subject. Since the problem, presented to the public in this manner, was to form the basis for this address, the idea occurred to me to buy several hundred copies of this magazine, distribute them at this meeting, and simply request everyone to read the article on page 73. The project, however, seemed prohibitively expensive and was discarded. This statement sounds familiar to those of us who have followed recent developments in the relation of medicine to society, and underlines an urgent and perplexing problem of great general concern. During the next few minutes I would like to review some of the major aspects of this situation and consider a few possible recommendations for the future that have stimulated the most attention and discussion.

Presidential address, Thirty-second Annual Meeting of the Central Society for Clinical Research, Chicago, Illinois, Nov. 6 and 7, 1959.

All of us engaged in research activities of any sort are obliged to divide our time and effort between the actual pursuit of scientific details of our experimental procedures, and a certain amount of worry about the perpetuation of our financial support. People who provide research funds have an annoying habit of wanting to know what use is being made of their money, and this function of periodic accounting by the recipient grows relentlessly as we progress, with the passing of years, from junior to senior investigators—and along with it comes, almost invariably, certain convictions and philosophical viewpoints concerning the sources, availability, and administration of the gigantic funds that support the entire structure of scientific research in American institutions.

During the past year, all of us in the medical sciences have been interested in, and appalled by, the content and implications of the now well known "Bayne-Jones report" sponsored by the Department of Health, Education and Welfare of the Federal Government, and the comparable report from the National Science Foundation, which deals with government-university relationships in federally sponsored scientific research and development. The trends for the future, which these reports portend, are so startling and even frightening that they are, of necessity, of great interest to every one of us.

Undoubtedly every generation has considered itself unique in terms of the magnitude of the crises it has faced, but it seems to me that even in the sober, dispassionate judgment of history, our present era will be looked upon as a "crossroads" of some sort, in regard to the integration of scientific research into the general stream of human life and progress. The accelerating speed of advancement of both basic and applied science functioning against the background of explosive social and population changes has resulted in the urgent need for a searching appraisal of our present status, and a plan for what appears to be a chaotic future. Any sense of complacency that we Americans may have had about our own scientific virtuosity was abruptly and rudely jolted by the launching of the sputniks, and by the gradual revelation that has followed of the true vitality and potential of the competitive research drive that exists behind the "iron curtain." Indeed, our whole educational system has been jarred to its foundations, and the resulting scrutiny of our objectives and methods has stimulated some sober thinking about our soft and easy way of life, and about the lack of discipline in our society.

What, then, is the true nature of our present dilemma in medical education and research? I would like to repeat a small amount of information which is now familiar to almost everyone. The Bayne-Jones report has indicated that we must expect to build and operate from 14 to 20 new medical schools by 1970, if we are to supply the 8,700 new physicians each year necessary to maintain the level of medical care that exists in this country at the present time. The major factor responsible for this critical situation is the explosion of population which threatens to give us a total census of 220 millions by that time. This population crisis, although generally known to most people, is not recognized in my opinion, to be as grave and ominous as it really is. The villain in the drama, incidentally, is none other than the medical scientist, who has learned to control one disease after another (especially those of childhood),

enabling more people to live much longer than could previously be expected. The factor of falling death rate is supplemented by an almost unbelievable human fecundity that would challenge the record of the most talented colony of rodents. In terms of research expenditure, the current level of nearly 350 million dollars annually is expected to rise to approximately one billion dollars by 1970. Even by the time of publication of the report, however, these estimates had been revised, and this expenditure is now expected to occur by 1967 or 1968.

The projection of thinking and planning one or two decades ahead on the scale indicated by these figures creates a number of specific and perplexing problems. The first of these concerns the supply of personnel to fill all the research positions that will be created by the expanding program. Those in the biologic sciences in general, and in the field of medicine in particular have not done a good job of "selling" their fields to high school students who are about to enter college. Those in the physical and engineering sciences, on the other hand, have manifested much more enterprise and resourcefulness in this program, and appear actually to have "stolen" significant numbers of youngsters who might otherwise have gravitated toward the biologic sciences. As testimony to this point of view, there has been a steadily falling number of medical school applicants during recent years, and some basic science departments in large universities find themselves with shrinking numbers of applicants for graduate study and the Ph.D. degree. Indeed, some departments faced with this dilemma have instituted advertising campaigns to bolster up their sagging enrollments. Since there can be no possible shortage of excellent potential candidates for graduate training in basic science, any attrition in applicants must be due to their diversion into other fields. Unless this trend is reversed, the projected expansion of medical research cannot possibly occur, regardless of the funds that may be available.

An equally disturbing trend has occurred in the recruitment of young men and women for medical training and practice. It is common knowledge that the ratio of applications to available places in most American medical schools has been falling over the past decade. With the sharp increase in need for physicians that lies ahead, this trend is hardly cause for complacency, and it is unlikely to be reversed unless we take some kind of positive action to change it.

A related problem is the degree and mode of participation of federal governmental agencies in the whole structure of scientific research in the United States. Upon this subject opinions vary from one pole to the other. Research these days is big business. It is remarkable and perhaps regrettable how few individuals are able, or at least content, to pursue high grade investigations at a relatively simple and modest level. Yet there are current examples of excellent contributions coming from such laboratories. The greater part of biologic and medical research, however, is carried on by "teams" of highly trained scientists using the most elegant and highly specialized equipment that becomes outdated almost before it has been put to use. This trend, of course, is perfectly legitimate and should be expected as a natural evolution of scientific

methods, but, at the same time, there is no need to equate bigness with excellence; indeed, I am familiar with some research settings in which data are turned out many fold faster than they can be interpreted and woven into the fabric of useful knowledge.

The huge program of research and training formulated in the Bayne-Jones report will require a sustained supply of astronomic amounts of money to make it function. Funds of such magnitude, in general, are not easy to come by, and the federal government is, in fact, the only possible, dependable source. In addition to providing stipends for thousands of investigators and trainees, the financing of an endless array of specialized, expensive equipment, and the purchase of routine laboratory supplies, the government agencies will be called upon for a larger and larger contribution to maintenance costs or "overhead" to the recipient institutions. Formerly at the 8 per cent level, this figure is now being raised to 15 per cent, with the probability that it will rise to 25 per cent in the near future. Agitation in Washington for 50 and 100 per cent allowance for indirect costs has been voiced in recent months, and the question so raised is not only of vital importance but involves some interesting implications. Some privately endowed universities have estimated their indirect costs of research at between 30 and 40 per cent, and the figure may vary from one institution to another. However, it is difficult to arrive at a fair and accurate ratio. Whatever the level of participation of the government in the basic operation of medical schools and universities, there can be no doubt that a higher figure than the current 15 per cent will be needed to avert a financial crisis in many institutions. Nevertheless, excessive financing of basic operations in medical schools must lead inevitably to a larger and larger stake in medical education as well as research by the government amounting, in effect, to an ultimate program of complete socialization of higher education. Under such conditions many of us wonder if the university can maintain its autonomy and remain free from undesirable control and inefficient administrative restrictions from Washington. Dr. Lowell Coggeshall from the University of Chicago and Dr. Lee DuBridge from "Caltech" have recently dealt very adequately with this matter in 2 important addresses.

A further problem relating to governmental research funds concerns the availability of money in excess of what can be used legitimately and wisely. Many laymen and even some scientists seem to have an idea, based in my opinion upon fallacious reasoning, that a crash program concentrating almost unlimited time, effort, brains, and money upon a single, well-defined problem must surely result in a solution in a reasonably short period of time. Such thinking has been applied, for example, to the cancer problem, on the theory that if enough people work on the problem, by the sheer force of mass action the answer must soon be found. It is my belief that this kind of reasoning is valid only to the extent that basic new scientific knowledge has not been put to practical use, and I doubt that many of our most pressing problems in clinical medicine can be solved in this manner. In any case, the application of such an approach leads, very naturally, to the bizarre phenomenon of funds being conferred upon one or many investigators who actually don't want more money and cannot use it. It must be emphasized, therefore, that there is a definite

finite ceiling to the appropriation of funds for scientific research—a ceiling which is geared to the numbers of able people available to make wise use of it, and the time they have to devote to the pursuit of their research. The administrative challenge is to find and maintain the optimal balance between supply and demand for money.

If the government is not to take over the entire business of medical research and education by virtue of its unique position as the sole source of adequate funds to maintain the expanding program, are there any other sources of money that may be considered of sufficient magnitude to produce any modification of the general situation? The structure of research support has changed strikingly in the past 2 decades. In 1940, the contribution of industry, philanthropy, and endowments was 14 times that supplied by the government. In 1957, the government's share was half again larger than that of the 3 other sources combined, with the disproportion increasing progressively year by year. Thus, in the foreseeable future all sources of funds other than government will shrink to relative insignificance.

This leads logically to the question of other possible donors of research money for the future. The big foundations, for the most part, are too heavily committed to other forms of philanthropy, including large investments in the social sciences. Nobody can possibly criticize such a policy. Private individual donations may occasionally provide supplementary help, but not in large amounts. Public money from solicitation for special disease research also may be helpful, but is hardly to be depended upon for sustained support. It seems quite clear, therefore, that industry is the only reasonable possibility. There are many large corporations which might logically have a stake in such an enterprise. Among these would be the pharmaceutical and life insurance companies, probably the chemical industry, and perhaps many more. Indeed, *all* industries might be interested. If each of 100 of these could be persuaded to contribute a large sum each year—say up to a million dollars (deductible from corporate taxes, of course) for 10 years, a hugh fund could be accumulated in a relatively short time. Such ideas are sure to give the most rugged industrial executive a "sinking spell." Good investment management of such a fund, however, would permit only the interest to be used for research grants on a large scale, and the support so provided could be perpetuated and increased indefinitely.

The matters that I have discussed are only representative facets of the larger problem that must be viewed through the perspective of the social revolution through which we are passing, the competitive political ideologies that are struggling for dominance, and above all, the unbelievably spectacular progress than man has made in understanding the world in which he lives and harnessing its forces for his own purposes. But time is running out. I have 5 recommendations that may be considered for the future:

1. Large institutional grants of money should be made by the federal agencies. This would simplify administrative detail by transferring this function entirely to the recipient institution. It would also minimize the attrition of funds in their journey from the taxpayer to Washington and back.

2. For the clinical sciences, more money should be made available for hospital beds. This is a form of support objected to by most donors, but vitally important to the area of applied research usually designated as "clinical investigation."

3. Arrangements should be made through government agencies, or recipient institutions, or both, to pay larger stipends to clinical investigators. In too many American institutions, clinical research is a part-time job supplemented, from economic necessity, by private patient consultation.

4. Possibility of creation of a large fund from industrial sources should be explored, as outlined previously, to augment the support derived from the government.

5. A large, carefully planned, energetic campaign of recruitment of high school and college students should be undertaken to provide the medical sciences with the manpower and womanpower of trained personnel, upon whom its life and vitality in the future will depend.

I wish to add one final personal philosophical comment. From the accumulated factual information upon which this discussion was based, I have a firm conviction that medicine and the biologic sciences which form its foundation are being swept along by an irresistable and relentless force into a pattern of complete socialization, the origin of which may possibly date back to 1932. Perhaps we must bear our share of the blame for this change. I think we, as doctors, have placed too high a premium on our services to society, in the form of fees which on the average are excessively high. We have fostered, or at least have acquiesced to a steadily rising cost of medical care for the average American that is now threatening the whole structure of our profession. Voluntary health and hospitalization plans have been of help and have delayed the final day of reckoning, but, for many millions of Americans, especially those in middle income brackets, these forms of assistance are still woefully inadequate, and serious injustices are being committed every day. The only answer, from the viewpoint of the average citizen caught in this trap, is some kind of a "national health act," whether it be patterned after the British plan, or some other. If the present trends continue, and I do not think we can change them, we may look forward to the federal government moving steadily into a position of virtually complete control of medical research, medical education, and medical care. I believe the direction of change is clearly marked for all to see, and if this prediction comes true, all supplementary or substitute forms of financing for these functions will be rendered unimportant or useless. Whether or not any one of us happens to favor or abhor this transition into "creeping socialism" the inescapable facts seem to point in that direction. Only the future can provide the answer.

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CLINICAL AND EXPERIMENTAL

RESULTS OBTAINED WITH THE ANTIGLOBULIN CONSUMPTION TEST AND INVESTIGATIONS OF AUTOANTIBODY ELUATES IN IMMUNOHEMATOLOGY

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IMMUNOHEMATOLOGY, a new field of scientific investigation, has developed in recent years. While a sufficient number of serologic methods for the investigation of erythrocyte antibodies and autoantibodies had been developed previously, the investigation of leukocyte and platelet antibodies was involved with methodic difficulties. The appearance of antibodies of the complete type (agglutinins) was demonstrated by Dausset,[1] Miescher,[2] Moeschlin,[3] Stefanini,[4] and Tullis.[5] From these investigations the conclusion was reached that platelet and leukocyte agglutinins appear in cases of idiopathic thrombopenic purpura (ITP) and idiopathic granulocytopenia (IGP). Just as in cases of acquired hemolytic anemia, it also proved possible to demonstrate the appearance of an autoagglutinin acting against the platelets of the patient in cases of ITP.[6] Transfusions of ITP blood or IGP blood caused a diminution of platelets or leukocytes in healthy recipients.[7-9] Injections of ITP or IGP serum caused marked thrombocytopenia or leukopenia in rabbits.[2, 8, 10] Further investigations showed that platelet immune antibodies arose after platelet transfusions in man[6] and that an experimental autosensitization was possible.[11]

In cases of hemolytic anemia, antibodies of the complete and the incomplete type were simultaneously demonstrable. In cases of ITP and IGP, one was generally confined to the investigation of complete antibodies (agglutinins). The demonstration of incomplete leukocyte and platelet antibodies by a routine technique seemed very desirable for the investigation of the immunopathogenesis of these diseases, and for support of the clinical diagnostic methods.

The Antiglobulin Consumption test, developed and published for the first time by us in 1954,[12, 13] seemed to be helpful in this situation. The Antiglobulin Consumption (AGC) test represents a method which, as a sequence of its technical properties, is especially qualified for the demonstration of incomplete antibodies. Further details about this characterization will be given later. The method may be performed in a direct form (demonstration of antibodies having become linked onto the cell surface in vivo) and in an indirect form (demonstration of circulating antibodies in the serum). This technique has

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been used with success by us for the demonstration of leukocyte antibodies[14, 15] and platelet antibodies.[16, 17] The demonstration of an autoantibody in cases of rheumatic fever and in cases of rheumatoid arthritis reacting with heart, e.g., connective tissue antigens,[12, 18, 19] in cases of liver cirrhosis[20] reacting with liver homogenizations, and in cases of multiple sclerosis[21] reacting with brain homogenizations was furthermore possible. The results of our investigation have been proved and confirmed by other investigators.[22-25, 28-30]

The principle of the test in its indirect form consists of incubating a pathologic serum simultaneously with a control serum with antigenic cells, e.g., leukocytes or platelets. These cells will become sensitized by antibodies which correspond serologically, when such antibodies are present in the pathologic serum. After eliminating all traces of nonspecifically adherent serum globulin from the cells by several washings, these cells, having become sensitized (experiment) or not sensitized (control), are brought into contact with an antiglobulin serum of known activity. Antibodies specifically linked onto the surface of the cells will react because of their globulin property with the antiglobulin antibodies. The antiglobulin serum pipetted off these cells will show decreased antiglobulin activity after consumption in contrast to the control, where no antiglobulin-consuming antibodies have been present. This decrease of antiglobulin activity gives an indirect sign of the antibody content having been present in the pathologic serum prior to incubation with the antigenic cells.

In the direct form of the AGC test, sufficient cells from the pathologic blood and from a control blood must be obtained to receive a satisfactory volume of a suspension of 40,000 leukocytes per c.mm. or of 1 million platelets per c.mm. With these cell suspensions, the test is carried out in the same manner as in the indirect form after the incubation of test cells with the pathologic serum.

MATERIALS AND METHODS

I. *Preparation.*—

A. *Preparation of leukocytes and platelets as antigen:* Leukocytes and platelets can be isolated individually from a blood of group O, Rh positive with the usual techniques. The cell sediments received are washed with saline and should be pure and not too intermixed with other cells. The method described by Maupin[26] has been proved to be very useful for the preparation of larger amounts of pure leukocyte and platelet sediments.

Cell sediments obtained from the blood of 4 to 10 donors (O, Rh positive) are collected and mixed. The total of the leukocyte or platelet sediment is lyophilized. To 4.0 ml. pathologic serum or control serum an amount of 10 to 20 mg. lyophilized leukocytes or of 20 to 40 mg. lyophilized platelets is added.

B. *Preparation of blood vessel homogenization:* Pieces of arterial and venous blood vessels are finely divided, washed, and homogenized. The mash is washed again with saline until the washings are clear. A suspension of the mash is filtered through a double layer of surgical gauze. The material passing through the gauze is collected, washed, and centrifuged. The cell sediment is collected and lyophilized. The lyophilized material is washed with ether and dried. Four milliliters of serum is incubated with an amount of 10 to 20 mg. lyophilized material.

II. *Performance of the Antiglobulin Consumption Test.* Forty milligrams lyophilized platelets or 15 mg. leukocytes are suspended in 4.0 ml. pathologic serum and control

serum. Each new preparation of platelets or leukocytes should be tested first with 3 to 4 control sera and 1 to 2 serologically positive sera to discover the best incubation ratio between cell weight (20 to 40 mg. platelets, 10 to 20 mg. leukocytes) and 4.0 ml. serum. The weight of cells which give strongly positive reactions, once determined, can serve for further experiments as standard incubation ratio for the cells of this preparation.

The mixtures of cells and serum are incubated in a water bath at 37° C. for 45 minutes. Then the suspensions are made up to 8.0 ml. with protein-free, cooled saline, are centrifuged, and the supernatant is discarded. The cell sediment is then washed 6 to 8 times with protein-free, cooled (+4° C.) saline. The supernatant of the last washing is pipetted off and the cell sediment is suspended with slight shaking into 1.0 ml., protein-free saline.

Several series of test tubes, each containing 5 protein-free tubes, are grouped together. Two drops of the antiglobulin serum, with a dilution which is 6 full titer steps less than the highest dilution which just gave a ± agglutination with the sensitized erythrocytes, are placed in the first tubes of all series. Ascending dilutions (2 drops) are put into the remaining tubes in each series, so that in the final tubes of each series the dilution of the antiglobulin serum is one titer step less than that giving ± agglutination in highest dilution with sensitized erythrocytes. For example, final antiglobulin titer is 1:1024±; dilutions used in the performance of the test are 1:32, 1:64, 1:128, 1:256, and 1:512. Two drops of the experimental cell suspension (cells which have been incubated with patient serum) are then dropped into each tube of the first series. The previously mentioned suspensions have to be slightly shaken before filling the tubes.

Now the filled tubes of the first series are shaken slightly for 45 seconds and are then centrifuged for a time long enough to permit them to be replaced into their appropriate series in the rack after exactly 3 minutes, inclusive of the time required for shaking and centrifuging at 3,000 r.p.m. The centrifuge used in this performance must be able to attain its maximum speed very quickly and to stop quickly.

One drop each of the supernatant antiglobulin serum is then quickly taken out of each tube and dropped onto a prepared protein-free glass plate in due conformity with the gradually increasing dilutions. The antiglobulin serum, once pipetted off the cell sediment after the consumption and placed onto the glass plate, shows no longer a change of its antiglobulin activity. In the routine performance of the test, working usually with 5 pathologic sera and 1 control serum for each sort of antigen, the incubation and the washings are done with all serum and control samples at the same time, while the consumption and the placing of the supernatant antiglobulin serum onto the glass plate is performed for one serum sample after the other. The control is investigated in the same way as described above as the last serum of the series under investigation. This performance facilitates the work with the test in the daily routine. Then the antiglobulin titer of the 5 different tests and of the control is recorded after the addition of sensitized erythrocytes.

Between the final titer of the antiglobulin serum prior to the consumption and the antiglobulin titer of the control after the consumption there is a difference of one-half or one titer step, for example, 1:1024± to 1:512+. Such results are recorded as negative. This difference results partly from a higher final dilution of the antiglobulin serum in the AGC test than in the titration of the antiglobulin serum prior to the AGC test, and partly from a slight nonspecific basic consumption by the surface of antigenic cells, especially in tests using tissue antigens. If larger differences between control test and original antiglobulin titer appear, they are caused by inaccurate washing and incompletely protein-free glassware. If no technical faults can be made responsible the control serum is to be regarded as false positive.

We use circular glass plates subdivided by 7 longitudinal and 7 latitudinal varnish lines, giving 6 longitudinal and 6 latitudinal rows with 6 square fields in each row. An example of an AGC test result is: First row (pathologic serum): dilutions of 1:32, 1:64, 1:128, giving ++ to ± agglutination, 1:256 and 1:512 being negative. Second row

(control serum): dilutions of 1:32, 1:64, 1:128, 1:256, and 1:512 giving +++ to ± agglutination. The AGC test result shows the difference between 1:512± and 1:128±, that is, a difference of 2 titer steps antiglobulin serum dilution.

III. *Preparation of Sensitized Erythrocytes.*—O, Rh positive erythrocytes are incubated with a standardized antiserum containing incomplete rhesus antibodies at the most favorable sensitization ratio[27] of 0.2 ml. packed erythrocytes + 3.0 ml. saline + 1.0 ml. antiserum at 37° C. for 30 minutes. These erythrocytes, sensitized with an incomplete rhesus antibody, made free of nonspecific serum proteins by washings, and suspended to a concentration of 7 to 10 per cent in protein-free saline, are used for all reactions with the antiglobulin serum.

IV. *Antiglobulin Serum.*—The antiglobulin serum used should be standardized. We use antiglobulin serum, Hemoderivate, Vienna, which shows regularly a final titer of 1:1024 to 1:512 with our sensitized erythrocytes.

V. *Isolation Technique.*—Lyophilized platelets or leukocytes are incubated with a pathologic serum (PS) and a normal serum (NS) under the same conditions as in the AGC test. When working with greater volumes of sera and using a previously determined incubation ratio (cells:serum), this ratio can be repeated several times with the purpose of obtaining a larger amount of isolated substances. In each incubation, 8.0 ml. PS or NS is mixed in a tube with 40 mg. lyophilized leukocytes or 80 mg. platelets. The cells are decentrifuged after incubation, the supernatant serum is pipetted off, and the remaining cell sediment is washed 8 to 10 times with protein-free, cooled (+4° C.) saline. The cell sediment of each tube is resuspended in 1.0 ml. protein-free, cooled, 0.9 to 2.0 per cent NaCl solution (elution liquid). The best results have been obtained with 2.0 per cent NaCl solution as elution liquid. The tubes, containing the cells suspended in the elution liquid, are put for 10 minutes into an ice bath and following this, into a water bath at 56° C. There they remain 20 minutes. After the first 10 minutes the tubes are taken out and are rocked for a short while. After the elution procedure the tubes are centrifuged 15 minutes at 3,500 r.p.m. and the supernatant liquid is carefully pipetted off the cell sediment. The elution liquids of several tubes are finally collected in one tube. From a 48 ml. volume of PS or NS, one receives, for example, an eluate of 6.0 ml. The eluates contain the isolated antibody and represent a material for further serologic, electrophoretic, and physiochemical investigations as well as for bioassays in animals.

VI. *Serologic Investigation of the Eluate.*—One drop of the eluate is placed on each of 6 square fields of the glass plate already described. One drop of antiglobulin serum in ascending dilutions is added, in such a way that the last drop of the eluate is mixed with 1 drop of an antiglobulin serum dilution 1 titer step below the dilution giving the final titer of the antiserum with sensitized erythrocytes. After 3 minutes' consumption time, 1 drop of sensitized erythrocyte suspension is added into each field. The agglutination is recorded. A difference between the agglutination of the eluate of NS and the eluate of PS is explained through antiglobulin consumption by globulins linked specifically onto the antigenic cells in the experiment and isolated into the eluate of PS. The result is recorded as the difference of titer steps of the final antiglobulin dilutions giving ± results in the control and the experiment.

A fundamental condition for the reliability of the AGC test as well as of the isolation technique is performance of the work after the incubation under conditions free of contaminating protein. All glass implements must be absolutely free of protein. Saline and elution liquids are considered as protein free, when composed of freshly bidistilled water and chemically pure NaCl. The test tubes and glass plates are made free of protein by brushing with soapy water, washing with bidistilled water, drying, depositing in chromium H_2SO_4 for 24 hours, washing in bidistilled water, and drying. Pipettes are made free of protein by washing with bidistilled water, depositing in chromium H_2SO_4 for 24 hours, washing with bidistilled water, and drying.

VII. *Animal Experiments With the Eluates.*—Control eluates and experimental eluates having a volume larger than 2.0 ml. are concentrated after dialysis to 2.0 ml. Control eluates and experimental eluates are injected in volumes of 2.0 ml. intravenously in the

ear vein of rabbits. The blood cells (erythrocytes, platelets, and leukocytes) of the animal are counted prior to the injection and 1, 2, 4, 6, 8, 48, 72, and 96 hours after the injection.

RESULTS

I. With the AGC test it was possible to demonstrate, for the first time, the appearance of an incomplete leukocyte antibody in idiopathic pancytopenia and idiopathic granulocytopenia. The results of our investigations concerning these diseases are recorded in Table I. Together with our results, we give the results of other investigators who have already used the AGC technique.

TABLE I. INVESTIGATION RESULTS OF THE ANTIGLOBULIN CONSUMPTION (AGC) TEST WITH LEUKOCYTES AS ANTIGEN

AUTHOR	CASES		TOTAL CASES	INDIRECT FORM OF AGC TEST		DIRECT FORM OF AGC TEST	
				+	−	+	−
Steffen	Serum of	Pancytopenia	15	7	8		
		Severe granulocytopenia	14	10	4		
		Chronic granulocytopenia	40	18	22		
		Aleukemic leukemia	4	−	4		
		Various diseases	32	−	32		
		Healthy individuals	30	−	30		
Dausset[25]	Leukocytes of	Leukopenia	51			22	29
		Chronic leukemia	12			−	12
		Various diseases	9			−	9
		Healthy individuals	45			−	45
Miescher[28]	Serum of	Pancytopenia	1	1	−		
		Granulocytopenia	1	1	−		
		Lupus erythematosus	7	7	−		
Rejholec[29]	Serum of	Granulocytopenia	17	13	4		
		Healthy individuals	20	−	20		

We investigated 135 sera and used leukocytes as antigen. Sixty-nine sera were taken from patients suffering from pancytopenia and granulocytopenia of varying severity. From 15 cases of idiopathic pancytopenia, 7 showed a positive and 8 a negative consumption result. From 14 patients with severe idiopathic granulocytopenia (leukocytes between 800 to 2,000 per c.mm.), 10 patients gave a positive and 4 a negative AGC test. From 40 patients suffering from idiopathic granulocytopenia in a chronic form (leukocytes between 2,000 and 3,500 per c.mm.), 18 patients showed a positive and 22 a negative result of the AGC test. Three cases of Felty's syndrome were among these 40 cases, giving 2 positive and 1 negative consumption reaction.

From the total of 35 sera reacting positively serologically, 14 cases had already received 1 or more transfusions prior to the serologic investigation. The antibody demonstrated in these 14 cases might be an isoimmune antibody or an autoantibody. Its reaction with leukocytes of various persons who are group O, Rh positive, makes it unlikely that it has the property of an isoimmune antibody. In the remaining 21 cases, transfusions were not performed prior to the serologic investigation. The assumption that this antibody is a leukocyte autoantibody, therefore, seems well justified.

The sera from 4 patients with aleukemic leukemia and 32 sera of persons suffering from various diseases (among them "labile" sera from cases of cancer, liver cirrhosis, γ-myeloma, and morbus Waldenström) gave negative consumption results. With the purpose of investigating the cell specificity of the leukocyte antibody, sera of 9 cases giving a positive AGC result with leukocytes as antigen were tested simultaneously with platelets as antigen. Eight sera reacted negatively with platelets; 1 serum gave a weak positive reaction (Table III). Thirty sera from healthy individuals, who were used as controls, always gave negative consumption reactions.

Dausset[25] obtained 22 positive and 29 negative results with the direct form of the AGC test in 51 cases of leukopenia. Since autologous leukocytes are used for antiglobulin consumption in the direct form of the test, the sensitization of the patients' leukocytes and the autoantibody nature of the leukocyte antibody was proved by these findings. Van Loghem[37] reports similar results. Leukocyte agglutinins were demonstrable simultaneously in the major part of Dausset's cases. In a total of 66 other cases, the test was negative. The findings of Dausett[22, 25] are important because they prove the simultaneous appearance of complete and incomplete leukocyte antibodies, whereby the leukocyte surface antigens are blocked by the incomplete antibodies for a reaction with circulating complete antibodies (agglutinins). These results explain the cause of the nonagglutinability of the patients' leukocytes in cases of pancytopenia and granulocytopenia by leukocyte agglutinins circulating in the blood. A major objection against an autoantibody nature of leukocyte antibodies in these cases seems to be refuted by these findings. Miescher,[28] working with the indirect form of the AGC test, observed a positive consumption result in 1 case of granulocytopenia and in 1 case of pancytopenia, using leukocytes as antigen. When using nuclei of leukocytes as antigen, strongly positive consumption results were obtained in 7 cases of lupus erythematosus. Rejholec[29] observed a positive indirect consumption test in 13 cases out of a total of 17 patients with idiopathic granulocytopenia, while sera of 20 healthy individuals gave negative results.

II. We have developed, in connection with the AGC test,[31, 32] a technique for the isolation of incomplete leukocyte or platelet autoantibodies (as well as for tissue autoantibodies). With the purpose of observing the difference in reactivity of the AGC test performed with serum and eluate, a series of 10 sera of patients suffering from idiopathic granulocytopenia, being more than a year in our serologic and hematologic control and showing repeatedly positive AGC tests, were used for the preparation of eluates in the following investigation. The results are recorded in Table II.

Column A gives the number of the cases; Column B shows the leukocyte count; and Column C the AGC test of sera 1 to 10 at the time of investigation. In all investigations, controls with a serum of healthy individuals were simultaneously performed and the AGC test results indicate the difference between the consumption of the pathologic serum and the control serum in titer steps of antiglobulin serum dilutions. Column D shows the incubation ratio between serum and leukocytes. Column E shows the volume of the eluates, and

Column F records the AGC test of the eluates, that is to say, the difference between the consumption of the eluate under investigation and of a control eluate in titer steps of antiglobulin serum dilutions.

Comparing the AGC test results of serum and eluate, one sees that in Cases 1 to 5 the results were closely related and an increase of the results in the eluate could not be obtained. In these experiments, we used a leukocyte suspension in saline with 20,000 leukocytes per c.mm. as antigen. In the experiments of Cases 6 to 10 we used dried and lyophilized leukocytes as antigen without suspending them previously in saline, suspended these lyophilized cells directly in the serum, and increased the incubation ratio by using more leukocytes against a volume of 4.0 ml. serum. In these cases the AGC test gave stronger positive results in the eluate than in the serum. A concentration of the autoantibodies in the eluates could be obtained. A certain excess of antigenic cells, adequate for the absorption of all antibodies in the serum, therefore, appears to be helpful for the increase of the test results. The use of an equal amount of antigenic cells in experiment and control is of importance.*

After incubation (incubation as a preparation for the elution experiment), 4.0 ml. portions of the sera (Cases 6 to 9) used previously for incubation, were incubated again with leukocytes and the AGC test was repeated. The results were negative. The leukocyte sediment, which remained behind after the elution procedure, was mixed under the usual conditions with an antiglobulin serum and was tested in respect to antiglobulin consumption. The tests were negative. These results indicate that (1) the autoantibody was quantitatively absorbed from the pathologic serum by the amount of leukocytes used, (2) the leukocytes were freed quantitatively from the autoantibody by elution, and (3) the globulins in the eluates giving positive AGC tests correspond to the autoantibody globulin of the pathologic serum.

III. The serologic results obtained with the AGC test proved the appearance of a leukocyte autoantibody in the serum of patients suffering from pancytopenia or idiopathic granulocytopenia. The results of the elution experiments proved that this antibody can be split off the surface of leukocytes,

TABLE II. COMPARISON BETWEEN THE RESULTS OF ANTIGLOBULIN CONSUMPTION (AGC) TESTS WITH SERUM AND ELUATE IN CASES OF GRANULOCYTOPENIA

A	B	C	D	E	F
CASE NUMBER	LEUKOCYTE NUMBER	AGC TEST SERUM	INCUBATION RATIO SERUM : LEUKOCYTES (L.)	VOLUME OF ELUATE	AGC TEST ELUATE
1	2,100	2	12 ml. + 2 ml. L. susp.	1.5 ml.	2½
2	2,600	2	12 ml. + 3 ml. L. susp.	1.0 ml.	2
3	2,500	3	12 ml. + 2 ml. L. susp.	1.5 ml.	3
4	2,800	1½	16 ml. + 4 ml. L. susp.	2.0 ml.	1
5	2,900	1	8 ml. + 2 ml. L. susp.	1.0 ml.	1
6	2,200	3	48 ml. + 300 mg. L.	8.0 ml.	4½
7	3,000	1½	32 ml. + 120 mg. L.	4.0 ml.	2½
8	1,500	1½	64 ml. + 280 mg. L.	8.0 ml.	3
9	3,000	2½	32 ml. + 160 mg. L.	4.0 ml.	4
10	2,200	2	32 ml. + 120 mg. L.	4.0 ml.	3

*In suspending 15 mg. lyophilized leukocytes in 4.0 ml. serum we received a suspension of about 20,000 leukocytes per c.mm. serum.

which have become sensitized by the antibody in a preceding incubation, and that this antibody can be isolated into NaCl solutions. These results did not prove that this antibody exactly represents the substance of the pathologic blood, which is responsible for the diminution of leukocytes in the course of idiopathic granulocytopenia.

Although observations have been reported that transfusion of IGP blood causes a diminution of the leukocytes in a healthy recipient[5, 9] and that injections of IGP serum or of an heterologous antileukocitic animal serum cause a marked leukopenia in animals,[2, 8] objections have been made against the

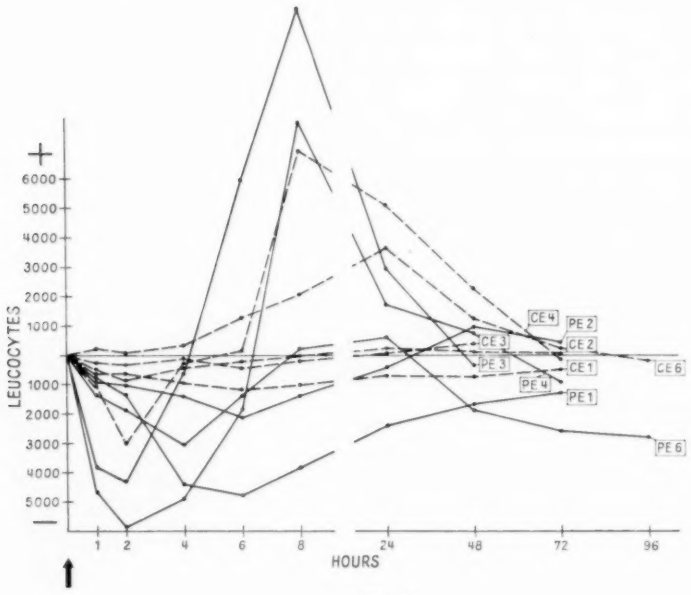

Fig. 1.

interpretation that the cause of this cell-diminishing effect is to be seen in a specific effect of leukocyte antibodies, since transfusions as well as injections of heterologous sera can be made responsible for a nonspecific transient cell diminution.[8, 33]

With the purpose of furnishing distinct evidence for the cell-diminishing effect of human leukocyte antibodies we prepared antibody eluates from IGP sera for bioassays. Control eluates were prepared simultaneously from control sera. A nonspecific effect of serum substances as the cause of cell diminution is to be excluded in these investigations, since the sensitized leukocytes were washed 8 to 10 times with protein-free saline after incubation with IGP serum and prior to the elution of the leukocyte antibody. All nonspecific serum substances are washed away by this treatment. In the eluates only, substances therefore appear which were split off the leukocytes by the elution procedure. Using these eluates in bioassays, one appears therefore to be well authorized to assume that every leukocyte diminution observed in these investigations must be caused by the leukocyte antibody

In investigating the cell count of leukocytes, platelets, and erythrocytes simultaneously, additional information about the cell-specific properties of the purified leukocyte antibody was to be expected. The eluates of Cases 1 to 4 and 6 (patients' eluate, PE) were injected intravenously in rabbits. The eluate of Case 6 was concentrated to a volume of 2.0 ml., the other eluates were injected in the volumes as indicated in Column E of Table II. Control eluates (CE) were injected simultaneously into other rabbits.

Fig. 1 shows a summary of the results of these animal experiments. With the purpose of making the drawing more distinct, the diminution or augmentation against the number of leukocytes per c.mm. prior to the injection was recorded instead of the different absolute leukocyte counts. After the injection of the eluates of Cases 1 and 2 (PE 1, PE 2) a significant decrease of the leukocytes appeared within 4 hours; this was followed by a leukopenia of 24 to 72 hours. After the injection of the control eluates (CE 1, CE 2), no significant change was apparent. After the injection of PE 3 and PE 4 (Cases 3 and 4), a significant decrease of the leukocytes appeared within 2 to 4 hours, which was followed by a significant increase of the cell number. After the injection of CE 3, no significant change of the leukocytes was apparent, while, when the control CE 4 was injected, marked leukocytosis appeared 6 hours after injection. Within 6 hours after injection of PE 6 (Case 6), a decrease of the leukocytes appeared which was normalized temporarily, reappeared after 24 hours, and lasted over the following 3 days. After the injection of the control (CE 6), a leukocytosis appeared, which was normalized after 48 hours. The erythrocyte and platelet counts, carried out simultaneously, showed no effect of the injections on the number of these cells and, furthermore, showed no difference between the cell counts after PE and CE injection.

IV. The AGC test has been proved to be a very useful technique for the detection of platelet antibodies. Our result in cases of ITP, in control cases, in controls with healthy individuals, and in cases of vascular purpura are recorded in Table III. The results of other investigators who used the same technique are added.

One hundred forty-two sera were tested by us with the AGC test using platelets as antigen. From the sera of 62 patients suffering from ITP, 41 sera gave a positive and 21 a negative consumption result. The platelet counts of the patients showed numbers between 18,000 and 150,000 cells per c.mm. Among the serologically positive cases were 29 adults and 12 children and among the cases serologically negative were 14 adults and 7 children. Five cases of leukemia (3 lymphatic and 2 myeloic forms) showed in 4 instances a negative and in 1 case a positive AGC result. The positive result was obtained with the serum of a patient suffering from lymphatic leukemia. Sera of 18 patients suffering from various diseases gave in all instances negative AGC tests. These sera consisted of sera from 5 patients with coagulation disorder (hypoprothrombinemia, PTA deficiency, hemophilia) and of 13 so-called "labile" sera from patients suffering from liver cirrhosis (5 cases), dysproteinemia (4 cases), pneumonia (2 cases), γ-myeloma (1 case) and morbus Waldenström (1 case).

TABLE III. INVESTIGATION RESULTS OF THE INDIRECT FORM OF THE ANTIGLOBULIN CONSUMPTION (AGC) TEST WITH PLATELETS, BLOOD VESSEL HOMOGENIZATIONS AND LEUKOCYTES AS ANTIGEN

AUTHOR	CASE	TOTAL CASES	AGC TEST					
			PLATELETS		BLOOD VESSEL		LEUKOCYTES	
			+	−	+	−	+	−
Steffen	Idiopathic thrombocytopenia	62	41	21	−	3	−	10
	Leukemia	5	1	4				
	Various diseases	18	−	18				
	Idiopathic granulocytopenia	9	1	8			9	−
	Hemolytic anemia	1	−	1			1	−
	Felty's syndrome	2	−	2			1	1
	Healthy individuals	35	−	35				
	Purpura rheumatica	5	−	5	3	2		
	Vascular purpura	3	−	3	2	1		
	Purpura fulminans	1	1	−	1	−		
	Periarteritis nodosa	1	−	1	1	−		
	Healthy individuals	12			−	12		
Moulinier[23]	ITP	7	7	−				
	Control patients	25	−	25				
	Healthy individuals	45	−	45				
Dausset[39]	ITP without transfusions	14	7	7				
	ITP with transfusions	31	19	12				

With the purpose of proving the cell specificity of the platelet antibody, 10 ITP sera, giving a positive consumption test with platelets as antigen, were tested simultaneously with leukocytes as antigen. These tests remained negative. Three ITP sera giving positive AGC results with platelets gave negative results when simultaneously investigated with blood vessel homogenizations as antigen. The sera of 35 healthy individuals gave negative results.

With the purpose of investigating the antigenic effect of platelets on leukocyte antibodies, 9 sera of IGP giving positive results with leukocytes as antigen were simultaneously investigated with platelets. These sera reacted negatively with platelets in 8 instances and gave a weak positive result in 1 case. One case of hemolytic anemia with a positive direct and indirect Coombs test reacted positively with leukocytes and negatively with platelets. Two cases of Felty's syndrome (1 positive, 1 negative with leukocytes) reacted negatively with platelets as antigen.

Using lyophilized platelets and lyophilized blood vessel homogenizations simultaneously as antigen, ten cases of hemorrhagic purpura were investigated. Five cases with the clinical diagnosis purpura rheumatica showed with platelets a negative AGC reaction. The same sera investigated with blood vessel homogenizations as antigen showed in 3 instances a positive and in 2 instances a negative AGC test. Three cases with the clinical diagnosis of vascular purpura showed with platelets negative and with blood vessel homogenizations in 2 instances a positive and in 1 instance a negative AGC result. The serum of 1 case of purpura fulminans reacted with platelets and blood vessel homogenizations positively. One case of periarteritis nodosa showed

TABLE IV. COMPARISON BETWEEN THE RESULTS OF ANTIGLOBULIN CONSUMPTION (AGC) TESTS WITH SERUM AND ELUATE IN CASES OF IDIOPATHIC THROMBOPENIC PURPURA

A	B	C	D	E	F
CASE NUMBER	NUMBER OF PLATELETS	AGC TEST SERUM	INCUBATION RATIO SERUM: PLATELETS (P.)	VOLUME OF ELUATE	AGC TEST ELUATE
1, a	60,000	2	32 ml. + 8 ml. P. susp.	4.0 ml.	2
b	30,000	2	48 ml. + 210 mg. P.	6.0 ml.	5
2, a	70,000	2	16 ml. + 4 ml. P. susp.	2.0 ml.	2
b	145,000	1	16 ml. + 4 ml. P. susp.	2.0 ml.	1
3	45,000	2½	64 ml. + 280 mg. P.	8.0 ml.	4
4	80,000	1	32 ml. + 140 mg. P.	4.0 ml.	1½
5	77,000	2	32 ml. + 140 mg. P.	4.0 ml.	4
6	18,000	2	48 ml. + 420 mg. P.	4.0 ml.	3½

with blood vessel homogenizations a positive, and with leukocytes and platelets a negative AGC result. Twelve sera of healthy individuals reacted negatively with blood vessel homogenizations as antigen.

In Rome in 1958, Dausset[30] recorded that 14 cases of ITP without transfusions gave 7 positive and 7 negative consumption tests and that 31 cases of ITP which had received transfusions prior to the serologic investigation gave 19 positive and 12 negative results with the indirect AGC test. Using the direct form as a supplement to the indirect form of the AGC test, it was possible to prove that these platelet antibodies appear in a circulating form in the serum of the patients as well as in a cell-bound form, linked onto the surface of the patient's platelets, sensitizing these platelets without agglutinating them. The incomplete type as well as the autoantibody nature of these platelet antibodies seems to be demonstrated by these findings.[25, 30] Moulinier[23] received with the same technique using the sera of 7 ITP patients, 7 positive reactions, and negative reactions with 25 sera of control patients and with 45 sera of healthy individuals.

V. Sera of 6 patients with typical ITP, who were a long time under our hematologic and serologic control and showed in repeated investigations a positive AGC test with platelets, were used in elution experiments. The serologic findings of these investigations are recorded in Table IV.

Column A gives the number of the case, Column B shows the platelet count, and Column C the AGC test of the sera 1 to 6 at the time of investigation. In all investigations, controls with sera of healthy individuals were simultaneously performed and the AGC test results are recorded as the difference between the consumption of the pathologic serum and the control serum in titer steps of antiglobulin dilutions. Column D shows the incubation ratio between serum and platelets. Column E shows the volume of the eluates, and Column F records the AGC test with the eluates, that is to say, the difference between the consumption of the eluate under investigation and a control eluate in titer steps of antiglobulin serum dilutions.

Comparing the AGC test result of the serum and of the eluate one sees in Cases 1, a, 2, a, and 2, b that the serologic findings were closely related. In these experiments a platelet suspension in saline of about 800,000 cells per c.mm. was used as antigen. Comparing the AGC test of serum and eluate

in Case 1, where different incubation ratios were employed, one sees that in the experiment 1,a using the platelet suspension as antigen an increase of the antibody concentration in the eluate was not obtained and the AGC test results of the serum and the eluate were identical. In experiment 1,b, weighed lyophilized platelets were put directly into the ITP serum (control serum) without being suspended in saline. Working with this incubation ratio, an increase of the AGC test result could be obtained in the eluate compared with the serum. A similar increase was observed in Cases 3 to 6. This increase is achieved by omitting a dilution of the serum during incubation and by using a higher amount of cells in the incubation ratio. The cell concentration of platelets after suspending 40 mg. dried platelets directly into 4.0 ml. serum results in 900,000 to 1,000,000 platelets per c.mm. serum. These can be counted individually and lie together with some little agglomerates of different numbers of platelets. An estimation of the average of the platelet concentration obtained in this incubation ratio, in our experience, a count of at least 1 million platelets per c.mm. serum. Everything said in paragraph II applies also for the observations described here. We are employing now as antigen only blood cells or tissue homogenizations in a dried and lyophilized state. The amount of these antigens being necessary for the incubation ratio of the test is measured by weight and the weighed antigenic substances are suspended directly in the serum designated for investigation.

After the incubation (incubation as a preparation for the elution), 4.0 portions of the ITP sera (Cases 1, 3, 5, and 6) which had been used previously for incubation were incubated again with platelets, and an AGC test was performed. The results were negative. The platelet sediment, which remained behind after the elution procedure, was mixed under the usual conditions with an antiglobulin serum and was tested with respect to antiglobulin consumption. The test was negative. These results indicate that (1) the autoantibody was quantitatively absorbed from the ITP serum by the platelets, (2) the platelets were freed quantitatively from the autoantibody through elution, and (3) the globulins in the eluates giving a positive AGC test correspond to the autoantibody globulin of the ITP serum.

VI. The serologic results obtained with the AGC test proved the appearance of an incomplete platelet antibody in the serum of the patients suffering from ITP. The results of the elution experiments proved that this antibody can be split off the surface of platelets, which have become sensitized by the antibody in a preceding incubation, and that the antibody can be isolated into NaCl solutions. These results did not give an exact proof that this antibody corresponds to the factor of the ITP blood or serum, which causes a diminution of the patient's platelets and which causes a decrease of the recipients' platelets after transfusions or injections of ITP blood or serum.

Although observations have been reported that transfusions of ITP blood cause a diminution of the platelets of a healthy recipient[7, 9] and that injection of ITP serum causes a marked thrombocytopenia in rabbits,[2, 10] objections

have been made against the interpretation that the cause of this cell diminution is to be seen in a specific effect of platelet antibodies[33] since transfusions as well as injections of heterologous sera can be made responsible for a non-specific transient platelet diminution.

With the purpose of furnishing distinct evidence for the cell-diminishing effect of human platelet antibodies, we prepared antibody eluates from ITP sera for bioassays. Control eluates were prepared simultaneously from control sera. Everything said in paragraph III about the specific effect of these eluates applies also to the investigations described in this section. The eluates of Cases 1, a, 2, a, 2, b, and 3 (patient eluate, PE), containing the in vitro reacting platelet antibody in an isolated state, were injected intravenously in rabbits. The eluate of Case 3 was concentrated after dialysis to a volume of 2.0 ml. The other eluates were injected in volumes as indicated in Column D, Table IV. Control eluates (CE) were injected simultaneously into other rabbits.

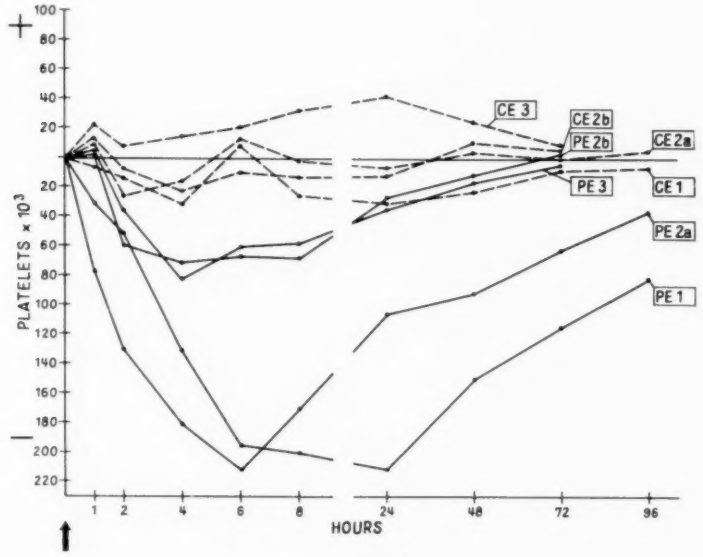

Fig. 2.

Fig. 2 shows a summary of the results of these animal experiments. With the purpose of making the drawing more distinct the diminution or the augmentation of the platelets (cells per c.mm.) in comparison to the platelet count prior to the injections was recorded instead of the absolute cell counts. After the injection of PE 1 from Case 1, a, a significant decrease of the platelets appeared within 4 to 6 hours, which was followed by a marked thrombocytopenia lasting more than 3 days. After the injection of the control CE 1, no significant change appeared in the platelet count. The PE of Case 2 was prepared twice. The first time on the peak of the thrombocytopenia (PE 2, a) and the second time at the beginning of convalescence with an increased platelet number and with diminution of the AGC test result (PE

2, b). After the injection of PE 2, a, a significant decrease of the platelets appeared again within 4 to 6 hours which was followed by a marked thrombocytopenia lasting more than 3 days. After the injection of PE 2, b, there appeared a less marked diminution of the platelets than in the experiment with PE 2, a. The thrombocytopenia was normalized after 2 days. After the injection of the controls CE 2, a and CE 2, b, no significant change in the platelet count appeared. After the injection of PE 3 (Case 3), a decrease of the platelets appeared within 4 to 6 hours, followed by a thrombocytopenia which was normalized after 3 days. The effect of this PE injection differed again significantly from the control (CE 3) in which a slight increase of the platelet counts appeared. The erythrocyte and leukocyte counts carried out simultaneously showed no effect of the injections on the number of these cells and showed furthermore no difference between the cell count after PE and CE injections.

DISCUSSION

With the AGC test and the elution technique described here, it was possible to demonstrate in the serum of patients suffering from idiopathic pancytopenia, granulocytopenia, and ITP a serum substance, which we designated as an incomplete leukocyte or platelet antibody. Methodically, the investigations were carried out as described in the section "Material and Methods." When working with blood cells as antigens another possibility of carrying out the AGC test consists in mixing the leukocytes or platelets after sensitization (incubation of 40 mg. lyophilized platelets or 15 mg. leukocytes in 4.0 ml. pathologic serum and control serum) and after several washings in their total amount in 1 tube with an antiglobulin serum of a dilution 6 full titer steps less than that just giving a ± agglutination with sensitized erythrocytes. After exactly 3 minutes including centrifugation and consumption time, the supernatant antiglobulin serum must be carefully pipetted off the cell sediment. With 0.1 ml. of this supernatant an antiglobulin serum titration with the sensitized erythrocytes can be carried out after the usual technique. The difference between the titration result of the pathologic serum and the control serum in titer steps is designated as the antiglobulin consumption of the pathologic serum.

The characterization antibody seems to be confirmed by the following cited properties: a specific serologic reactivity for leukocytes or platelets could be proved in vitro. Investigations simultaneously performed with leukocytes and platelets as antigen showed that the antibody appearing in ITP sera reacted with platelets and did not react with leukocytes, while the antibody appearing in IGP sera reacted with leukocytes and did not react with platelets. It was furthermore demonstrable that the antibody appearing in ITP sera could be absorbed by platelets and not by leukocytes, while the antibody appearing in IGP sera could be absorbed quantitatively by leukocytes and not by platelets. The antibody linked specifically onto leukocytes or platelets could be eluted quantitatively from these cells by heating (56°

C.) and could be demonstrated serologically in the eluates. An incubation of the antibody isolated in the eluates together with leukocytes or platelets as antigen resulted in a positive AGC test with leukocytes, when the antibody was isolated from an IGP serum, and a positive AGC test with platelets, when the antibody was isolated from an ITP serum. In further investigations[35] we were able to prove that the leukocyte or platelet antibody, having become purified by elution, emigrates electrophoretically with its major part like γ-globulin, while a small amount emigrated in a zone of β_2 annexed to the γ-globulin zone. Immunoelectrophoretically the antibody is precipitated by anti-γ-globulin serum, while antialbumin, anti-α, and anti-β-globulin serum cause no precipitation. Ultracentrifugal studies showed that the leukocyte and platelet antibody possess a S_{20} of 7.0 to 7.5.

In a further classification of these antibodies we designated them as autoantibodies. The autoantibody nature of these antibodies has been proved by the simultaneous performance of the direct and indirect AGC test. Using the direct form as a supplement to the indirect form it was possible to demonstrate that platelet or leukocyte antibodies appear simultaneously in a circulating form in the serum and in a cell-bound form, being linked onto the surface of the platelets or leukocytes of the patient and sensitizing these cells without agglutinating them. These investigation results possess a certain parallelism to the results of the direct and indirect Coombs test in acquired hemolytic anemia.

The direct form of the AGC test detected antibodies linked onto the surface of leukocytes or platelets of the patient, which sensitized these cells but did not agglutinate them. Agglutination tests and the AGC test, simultaneously performed, showed, in a certain number of cases,[30, 38] negative results of the leukocyte or platelet agglutination test and a positive result of the AGC test. The platelet or leukocyte antibodies, isolated in our elution experiments, were able to sensitize in a renewed incubation platelets or leukocytes of O, Rh positive donors but did not agglutinate them. These findings point to an incomplete antibody type.

The consumption of antiglobulin antibodies is performed in the AGC test, after the antigen antibody reaction (for example, combination of platelets and platelet antibody) has taken place. The antigen antibody complex after the removal of nonspecific serum proteins by several washings is in reality the substance which is investigated in regard to antiglobulin consumption. Assuming that antigen antibody complex consists of cells sensitized by an antibody, the globulin property of these antibodies should be wholly available for a reaction with antiglobulin antibodies. Antibodies, being however in their major part fixed in the interior of the network of an antigen antibody complex, such as for example, an agglutinate, seem to be not easily available with all their globulin determinants for a reaction with antiglobulin antibodies. We believe, therefore, in evaluating the type of antibody, which in general is detected by the AGC test, that one should say that antiglobulin consumption will occur with the globulin property of complete and incomplete antibodies,

but, as a result of the technical characteristics of the test, the AGC technique demonstrates especially in the case of blood cell antibodies an incomplete antibody type.

In the course of our investigations we were able to make the following observations about the properties of these antibodies: complement must not necessarily exist for their reaction with leukocytes or platelets, since the AGC test can be accomplished with inactivated sera too. On the contrary, an inhibiting substance seems to be present in fresh sera since the AGC results become more distinct with 2- or 3-day-old sera than with freshly drawn sera. The serologic activity of the sera remains unchanged during several weeks' storage in the refrigerator. The most favorable area of reactivity is present at 37° C. An elution succeeds at a temperature of 56° C. as well into solutions of 0.9 to 4.0 per cent NaCl (2.0 to 4.0 per cent NaCl giving more satisfactory results) as into Veronal-Na and KH_2PO_4-borax buffer of a pH ranging from 6.4 to 8.8. Dausset[36] confirms these observations and states that these antibodies are not absorbable by $BaSO_4$ or $Ca_3(PO_4)_2$.

Transfusion experiments in human beings and animal experiments with injections of ITP or IGP serum proved that blood or serum of ITP or IGP patients causes a diminution of leukocytes or platelets. The results of the animal experiments described in this paper prove that a serologically demonstrable leukocyte or platelet antibody possesses, in a purified state, the property of diminishing quickly the leukocyte or platelet count of rabbits, and of causing a leukopenia or thrombopenia of several days' continuance.

The specificity against platelets or leukocytes of this effect is proved both by the exclusive diminution of the cells which correspond serologically to the isolated antibody, as well as by the negative effect of the injection of control eluates.

Animal experiments of Miescher,[2] Moeschlin,[8] Pfeiffer,[10] and others prove that leukocytes and platelets of rabbits react with leukocyte or platelet antibodies of man and confirm the justification for the use of rabbit blood cells in our investigation.

Whereas in animal experiments with isolated platelet antibodies a cell-diminishing effect appeared exclusively, there appeared in the experiments with isolated leukocyte antibodies, together with the specific cell-diminishing effect, a supplementary, nonspecific, leukocyte-augmenting effect in 2 animals after injection of antibody eluates, as well as in 2 control animals. We believe that in these experiments a supplementary, nonspecific factor was eluted from the surface or from the interior of the leukocytes similar to Menkin substances which seems to be responsible for the leukocytotic effect of the eluates.

The animal experiments show furthermore that the effect of the platelet antibody eluates had a quantitative character. The platelet-decreasing effect of an eluate (Case 2, a), prepared at the time of maximal thrombocytopenia, was very significant within 6 hours after injection and was followed by a thrombocytopenia in the animal lasting more than 4 days. In contrast to

this observation, an eluate of the same case (Case 2, b), prepared at the beginning of convalescence, showed a less indicative effect within the first 6 hours and the following thrombocytopenia was normalized after 3 days. We believe that a difference in the amount of antibodies present in the original sera and in the corresponding eluates is responsible for these observations.

A review of the relations between the appearance of the clinical symptoms of ITP or IGP and a positive result of the AGC test shows that the test gave positive results in 66 per cent of ITP, in 50 per cent of idiopathic pancytopenia and, depending on the severity of the illness, in 45 to 71 per cent of idiopathic granulocytopenia. Sera of patients suffering from other diseases and especially so-called "labile sera" as well as sera of healthy individuals gave, in all instances, negative results of the AGC test with platelets and leukocytes as antigen. The AGC test seems, therefore, to be a reliable method for the diagnosis of immunothrombopenia and immunogranulocytopenia. In comparing the course of ITP or IGP with the results of several AGC tests, having been performed during longer periods of illness, 2 types of reaction patterns could be observed in general. In a certain number of cases the AGC test became negative together with a clinical improvement of the patient. Two cases may be mentioned as examples: (1) B. A., a female, IGP, October, 1958, AGC 4 titer steps, leukocytes 1,000; January, 1959, AGC test 2, leukocytes 2,400; April, 1959, AGC negative, leukocytes 4,300. (2) W. L., a male, ITP, Aug. 1, 1958, AGC 2½, platelets 16,000; Aug. 29, 1958, AGC 2, platelets 18,000; October, 1958, AGC 1, platelets 80,000; December, 1958, AGC negative, platelets 168,000. Other cases showed together with an undulating course of the illness and without a significant clinical improvement sustained positive results of the AGC test. Two cases will again be mentioned as examples: (1) Z. H., a female, IGP, September, 1957, AGC 2, leukocytes 2,200; January, 1958, AGC 3, leukocytes 1,800; April, 1958, AGC 2, leukocytes 2,200; September, 1958, AGC 2, leukocytes 2,200; December, 1958, AGC 2, leukocytes 1,700; April, 1959, AGC 2, leukocytes 2,100. (2) K. F., a male, ITP, August, 1956, AGC 2, platelets 30,000; December, 1956, AGC 1½, platelets 45,000; January, 1957, AGC 2½, platelets 45,000; October, 1957, AGC 2, platelets 31,000; June, 1958, AGC 1½, platelets 39,000; January, 1959, AGC 3, platelets 30,000.

In investigating sera of patients suffering from vascular purpura simultaneously with platelets and blood vessel homogenizations as antigens, the findings indicated that a blood vessel antibody appears in cases of vascular purpura and support the opinion that vascular purpura possesses an immunological origin. Miescher[28] used the AGC test with leukocyte nuclei as antigen for the investigation of cases of lupus erythematosus and found a positive result in all cases investigated. Dausset[36] succeeded in demonstrating with the AGC test the antigen D onto leukocytes and platelets. Van Loghem[38] reported that the AGC test enables a differentiation between leukocyte isoantibodies and leukocyte autoantibodies, when using papain-treated leukocytes as antigen. Papain treatment shall abolish the antigenicity of leukocytes for isoantibodies, while their antigenicity for autoantibodies remains unchanged after papainization.

In concluding the discussion we want to state that the experiments described regarded in their entirety prove with a high degree of probability that leukocyte or platelet autoantibodies appearing in ITP or IGP possess a cell-diminishing effect. Therefore one should attribute to them a causal influence on the pathogenesis of ITP and IGP, and autoaggression as an immunopathogenetic principle[34] appears to be responsible for the development of these diseases. We therefore use the collective term of autoaggressive disease for them. From this immunopathogenetic principle we are beginning to know about the second stage: the existence of autoantibodies, their noxious influence on certain cells, their specific appearance in the blood of patients suffering from different diseases, which in common possess a still unknown pathogenesis, a chronic and undulating course with relapses of different severity caused by different and nonspecific stimulants. In accordance with our concept of autoaggression[34] it seems understandable that the nonspecific irritations of such different causes of relapses lead to a nonspecific stimulation of renewed autoantibody production (anamnestic response) which has already been introduced by earlier autosensitization. These causes of relapse appear therefore as nonspecific irritants of a hidden specific mechanism, the autoantibody production with consequent autoaggression. The first stage of this mechanism, the way of autoantigenization of cells by certain pernicious causes (influence of virus, bacteria, etc.) in man, the fate of these autoantigens, and the intensity and continuance of the development of autoantibodies following these autoantigenic stimuli remain still unknown. We believe, however, that the investigation results about autoaggression obtained until now point on the one hand with likelihood to the existence of this immunopathogenetic principle, and that they represent on the other hand a challenge to further research into its mechanism.

SUMMARY

1. The Antiglobulin Consumption test is described. Results obtained with this technique are shown. One hundred thirty-five sera were investigated using leukocytes as antigen. Depending on the severity of illness 45 to 71 per cent of the sera investigated of patients with idiopathic granulocytopenia and pancytopenia gave positive results, while sera of control patients and healthy individuals gave negative results.

2. Using platelets as antigen, we investigated 142 sera. Sixty-six per cent of the sera investigated of patients with idiopathic thrombopenic purpura gave positive results, while sera of control patients and of healthy individuals gave negative results. In comparative investigations of sera of patients with vascular purpura with platelets and blood vessel homogenizations as antigen the experiments with platelets gave negative results, whereas with blood vessel homogenizations in one-half of the sera investigated positive results were obtained.

3. An elution technique for the isolation of leukocyte and platelet antibodies is described. The serologic investigation results of the eluates, proving the appearance of the antibody in the eluates, are recorded.

4. Animal experiments are described in which antibody eluates were injected intravenously in rabbits. Depending on the cell specificity of the isolated antibodies, a significant diminution of leukocytes or platelets took place followed by a thrombocytopenia or leukopenia lasting several days. Injections of control eluates did not produce such effects.

5. The serum factor responsible for a positive Antiglobulin Consumption test, elutable from cells onto which it has become linked specifically and responsible for a cell-diminishing effect of the eluates in animal experiments, is characterized as a platelet or leukocyte autoantibody. Its significance for the development of thrombocytopenia and granulocytopenia, as well as autoaggression as a pathogenetic principle, is discussed.

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THE DISTRIBUTION OF Co^{58}-LABELED VITAMIN B_{12} IN THE RAT WITH FATTY METAMORPHOSIS AND CIRRHOSIS OF THE LIVER

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THE cirrhotic liver has been found to contain a smaller amount of vitamin B_{12} than the normal liver,[1] while the plasma level of vitamin B_{12} in the cirrhotic patient is elevated.[2, 3] It has been assumed that this abnormality is due to cell injury and to release of the cell content into the circulation. This mechanism appears to be responsible for the high plasma levels of cyanocobalamin in infectious and toxic hepatitis where extensive necrosis is characteristic. Necrosis is a minor feature of uncomplicated chronic nutritional cirrhosis. It is possible that the binding capacity of the cirrhotic liver for vitamin B_{12} is diminished. This may lead to the utilization of other potential binding sites, such as the plasma and the kidney.

The rat appeared to be a suitable experimental animal for the study of this problem. Experimental cirrhosis can be produced regularly in the rat by dietary measures. Furthermore, the distribution of Co^{60}-cyanocobalamin in the normal rat has been studied extensively.[4-7] The present investigation was designed to study the distribution of radioactive cyanocobalamin in the rat with fatty metamorphosis and cirrhosis of the liver.

METHODS

Several groups of approximately 1-month-old Sprague-Dawley rats were maintained on a high fat, low choline diet* consisting of 48 per cent glucose, 40 per cent lard, 5 per cent casein, 5 per cent salt mixture and a vitamin fortification mixture with the omission of choline chloride and inositol. The vitamin B_{12} content of the diet was 1.35 mg. per 100 pounds. This high fat, low choline diet will be referred to as "HFLC diet."

There was an approximately equal number of control animals which were maintained on a normal rat diet (Purina Chow). Each animal received 1 subcutaneous injection of Co^{58}-cyanocobalamin (specific activity 0.3 to 0.5 μc per mcg.) at a dose of 1.3 mcg. per 100 Gm. of body weight.[7] Following the injection, urine was collected for 48 hours. The schedule of the injections and the time of the subsequent sacrifice of the animals in each group was as follows:

1. Fourteen animals received the injection on the thirty-eighth day of the experiment. Seven of these (Group A, Table I) received the HFLC diet and the others received the control diet (Group B, Table I). They were killed on the fortieth day.

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*Special Diet S. L. H. (Nutritional Biochemicals Corporation, Cleveland 28, Ohio).

TABLE I. CO^{58}-VITAMIN B_{12} CONTENT IN LIVER AND KIDNEYS OF RATS ON A HIGH FAT, LOW CHOLINE DIET

GROUP	NUMBER OF ANIMALS	LIVER							KIDNEY			
		PER CENT FAT CONTENT	PER CENT DOSE	P VALUE	MμG PER GRAM	P VALUE	MμG PER GRAM FAT-FREE	P VALUE	PER CENT DOSE	P VALUE	MμG PER GRAM	P VALUE
A. Forty days* (2 day test)	7	32.2 ± 9.6	3.16 ± 0.59	$p < 0.4$	7.31 ± 1.9	$p < 0.01$	10.8 ± 2.0	$p < 0.1$	16.2 ± 1.92	$p < 0.01$	233.0 ± 33	$p < 0.6$
B. Control	7	4.6 ± 0.95	2.75 ± 0.75		13.0 ± 2.9		13.6 ± 3.1		11.54 ± 2.60		222.0 ± 37	
C. Forty days† (14 day test)	7	25.2 ± 9.5	2.24 ± 0.42	$p < 0.01$	4.61 ± 2.0	$p < 0.01$	6.02 ± 1.9	$p < 0.01$	15.10 ± 4.60	$p < 0.01$	213.0 ± 68	$p < 0.01$
D. Control	7	4.25 ± 0.28	4.49 ± 1.10		13.2 ± 3.6		13.8 ± 3.8		5.02 ± 1.58		72.0 ± 6.8	
E. Six months‡ (14 day test)	14	7.37 ± 3.5	2.55 ± 0.69	$p < 0.2$	8.28 ± 2.9	$p < 0.01$	8.60 ± 2.5	$p < 0.01$	15.92 ± 1.60	$p < 0.01$	188.0 ± 34	$p < 0.01$
F. Control	20	4.26 ± 0.63	2.94 ± 0.66		13.4 ± 3.4		14.0 ± 3.7		6.54 ± 1.64		113.0 ± 25	

*Rats were on a high fat, low choline diet for 40 days. They received Co^{58}-vitamin B_{12} (1.3 μg per 100 grams of body weight) on the thirty-eighth day of the dietary regimen. They were sacrificed 2 days later.

†Rats were on a high fat, low choline diet for 40 days. They received Co^{58}-vitamin B_{12} (1.3 μg per 100 grams of body weight) on the twenty-sixth day of the dietary regimen. They were sacrificed 14 days later.

‡Rats were on a high fat, low choline diet for 6 months. They received Co^{58}-vitamin B_{12} (1.3 μg per 100 grams of body weight) 14 days prior to sacrifice. The listed values indicate means (± standard deviation).

2. Fourteen animals were injected on the twenty-sixth day of the diet and were sacrificed 14 days later. Half of this group (Group C, Table I) received the HFLC and the other half (Group D, Table I) received the normal diet.

3. It had been planned to maintain 20 animals on the diet for 6 months; only 14 animals survived. They received the injection of Co^{58}-cyanocobalamin 14 days prior to sacrifice (Group E, Table I). Twenty animals on a control diet (Group F, Table I) were treated in the same fashion. One of these animals had died before the completion of the study.

4. Sixteen rats from a different litter were used to study the Co^{58}-cyanocobalamin content of the liver and kidney, and also of the remaining carcass. Nine of these animals had received the HFLC diet for 5½ months and 7 had received the normal diet. They were injected with Co^{58}-cyanocobalamin 2 days before sacrifice. Following the injection, the urinary excretion of Co^{58}-cyanocobalamin was measured in a 48 hour sample (Table III).

The animals were killed by a blunt blow to the head and were then dissected. In all animals the Co^{58}-cyanocobalamin content was determined in the liver and kidney, and in the animals of Group IV this was also done in the remaining carcass. The following method was used.

The liver and the right kidney were removed, rinsed with physiologic saline, blotted dry, and weighed. A sample from each organ was taken for histologic examination and one or more additional sections were placed in previously weighed 25 ml. volumetric flasks where the tissue was dissolved in concentrated KOH solution. The entire flask was placed directly on top of a scintillation well crystal, with adequate shielding. Sufficient counts were obtained to yield a standard deviation of ±3 per cent or less for each count.*

The Co^{58}-cyanocobalamin content of the liver and kidney was expressed (1) as per cent of the injected dose, (2) as millimicrograms per gram of tissue, and (3) as millimicrograms per gram of fat-free tissue in case of the liver. The fat content was determined by extracting and weighing the fat according to the method of Folch, Lees, and Sloane Stanley.[8]

The urinary excretion of Co^{58}-vitamin B_{12} was determined by diluting the collected urine to 60 ml., removing a 3 ml. aliquot, and comparing its activity with that of a suitable standard in the scintillation well counter.

All statistical analyses were performed with the Student "t" method.

RESULTS

The animals which received the HFLC diet failed to gain weight as was expected (Fig. 1). By the fortieth day, all animals had developed severe fatty metamorphosis of the liver, with an average fat content of 28.7 per cent (Table I). The fat content of the liver of the control animals averaged 4.4 per cent. At 6 months, the HFLC-dieted animals had developed nutritional cirrhosis of varying severity with an average liver fat content of 7.4 per cent.

In the animals which had received the HFLC diet for 40 days, the percentage of administered Co^{58}-cyanocobalamin retained in the liver 2 days following injection (Table I, Group A) was approximately the same (3.2 per cent) as in the control animals (2.8 per cent) (Table I, Group B). Two weeks following the injection, however, the liver of the control animals contained, on the average, 4.5 per cent of the administered dose (Table I, Group D). During the same period, the HFLC-dieted animals retained in the liver only 2.2 per cent of the dose (Table I, Group C). These differences were even greater when the Co^{58}-cyanocobalamin content was not expressed as per cent of the administered dose,

*This method of counting was found to be superior to the counting of small aliquots, as an increase of 154 per cent in the counting rate was obtained. This method also requires fewer calculations and eliminates pipetting. The counts were corrected for background and compared with the corrected counts of suitable standards prepared from an aliquot of the injected stock solution.

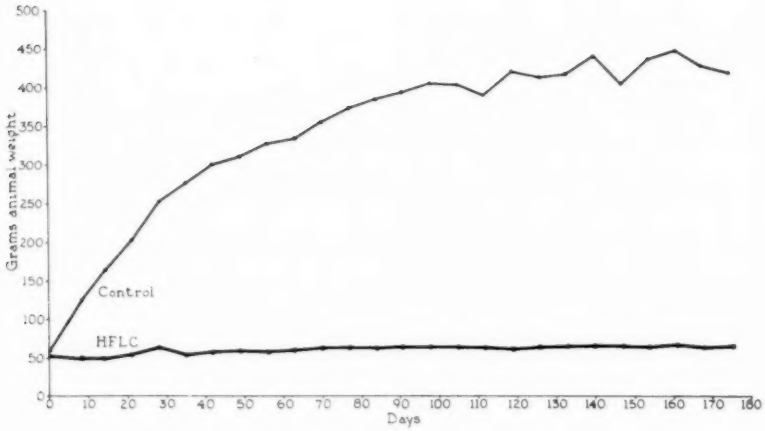

Fig. 1.—Weight curve of rats on normal and HFLC diet.

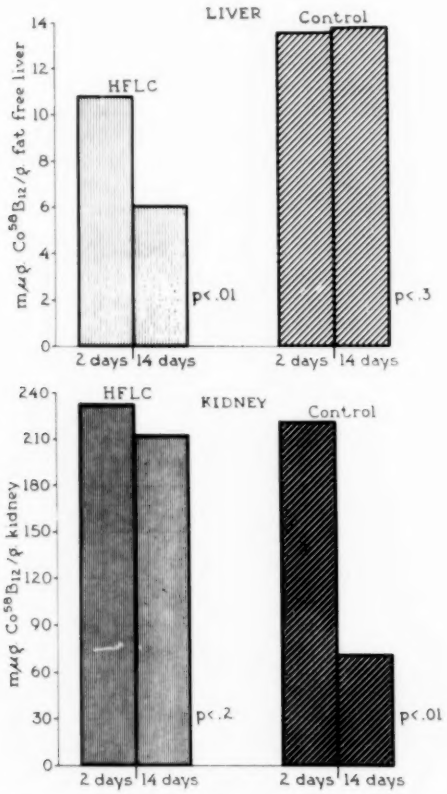

Fig. 2.—Co^{58}-cyanocobalamin concentration in liver and kidney, 2 days and 14 days following parenteral administration.

but as actual content in mμg per gram of liver tissue. There also was a significant difference between the Co^{58}-cyanocobalamin content in the liver of the HFLC-dieted animals 2 days and 14 days following injection (Fig. 2). The liver of the latter group of animals contained approximately 40 per cent less than the liver of the animals which were sacrificed on the second day. In the normal rat, however, the content of Co^{58}-cyanocobalamin per gram of liver was approximately the same on the second as on the fourteenth day.

In the rats which were maintained on the diet for 6 months and in the corresponding control animals, only the 14 day test was completed (Table I, Groups E and F). The percentage of the administered dose found in the liver was insignificantly higher in the control than in the HFLC-dieted animals. On the other hand, a statistically significant difference was present between the actual Co^{58}-cyanocobalamin concentration of the normal and the cirrhotic liver.

It is of interest that in all control animals, the amount of Co^{58}-cyanocobalamin per gram of tissue was approximately the same, despite the marked change in body weight and in the size of the administered dose from the fortieth to the one hundred-eightieth day. It was considered possible that these figures indicated the existence of a state of saturation of the liver for vitamin B_{12}. This hypothesis was tested by injecting different amounts of Co^{58}-vitamin B_{12} into healthy adult rats and measuring the labeled vitamin in the liver 2 days later (Table II). The administered doses per 100 grams of body weight were 0.66,

TABLE II. DEPENDENCE OF CO^{58}-CYANOCOBALAMIN CONTENT OF LIVER AND KIDNEY ON SIZE OF ADMINSTERED DOSE

DOSE PER 100 GRAMS BODY WEIGHT	NUMBER OF ANIMALS	LIVER			KIDNEY		
		PER CENT DOSE	MμG PER GRAM	MμG PER GRAM FAT-FREE	PER CENT DOSE	MμG PER GRAM	URINE PER CENT DOSE
0.66 meg.	7	3.29 ± 0.52	7.12 ± 1.2	6.86 ± 1.2	9.68 ± 2.1	163 ± 40	33.8 ± 15.0
1.32 meg.	6	2.04 ± 0.48	8.67 ± 1.7	8.35 ± 1.7	6.96 ± 1.0	198 ± 20	44.0 ± 16.0
2.64 meg.	6	1.51 ± 0.65	11.30 ± 4.6	10.88 ± 4.6	4.43 ± 0.9	278 ± 47	63.6 ± 11.0
5.28 meg.	7	0.95 ± 0.18	15.50 ± 3.5	14.93 ± 3.5	2.76 ± 0.3	342 ± 33	68.7 ± 8.8

The data are expressed by means (± standard deviation).

1.32, 2.64, and 5.28 meg. Six or seven animals were in each group. While the hepatic uptake expressed as per cent of the administered dose diminished with the increase in this dose, the Co^{58}-vitamin B_{12} content of the liver, expressed as mμg per gram of liver, increased in an almost linear fashion with successive increases in the dose (Fig. 3). Saturation was not reached at the dose levels of the experiment.

The Co^{58}-cyanocobalamin concentration of the liver in the animals which had received the HFLC diet for 6 months (Table I, Group E) was higher than in the younger animals with fatty metamorphosis (Table I, Group C). The difference was statistically significant ($p<.01$). This was also true when the values were calculated as to express the Co^{58}-vitamin B_{12} content per gram of fat-free tissue. The frankly cirrhotic liver, containing less than 10 per cent fat, had a greater binding capacity for Co^{58}-cyanocobalamin than the liver with severe fatty metamorphosis.

The difference in the cyanocobalamin binding capacity of the kidney between the normal and the HFLC-dieted animal was as marked as in the case of the liver, but in the opposite direction (Table I, Fig. 2). The lower the binding capacity of the liver, the greater was the apparent concentration of Co^{58}-cyanocobalamin in the renal parenchyma. The kidney of the HFLC-dieted animal, therefore, retained a larger amount of the vitamin than the normal. This was the case whether the values were expressed as per cent of the administered dose, or as mμg per gram of tissue. Most striking was the difference between the 2 groups sacrificed 2 weeks following injection. By that time, the kidneys of the HFLC-dieted animals retained approximately 3 times more than the normal (Fig. 2).

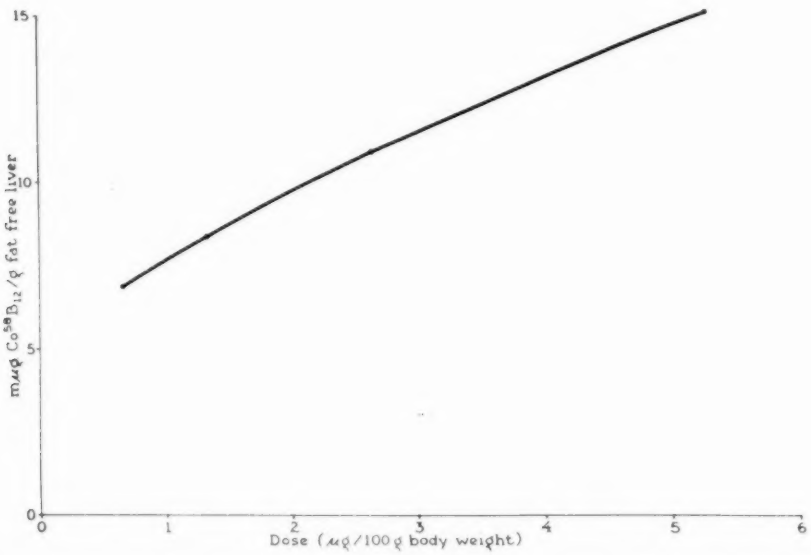

Fig. 3.—Dose-hepatic concentration relationship of Co^{58}-cyanocobalamin (see Table II).

The sixteen rats of Group IV were given the HFLC diet for 5½ months. At the end of this period, they were sacrificed 2 days following the injection of Co^{58}-cyanocobalamin. It was planned to compare this group with animals of the same litter which were to receive the injection 14 days prior to sacrifice. Unfortunately, for unknown reasons, the mortality of the latter group of animals was so high that no valid observations could be made. These 16 rats were then used for the determination of the Co^{58}-vitamin B_{12} content of the liver and kidney and the remaining carcass. The figures for liver and kidney were similar to the previously described group of cirrhotic animals (Table I, Group E) which were examined 14 days following the injected dose. In the remaining carcass, there was no difference in the percentage of the retained labeled vitamin between the HFLC-dieted and control animals (Table III). In the control animals, 91.5 to 100 per cent of the administered dose could be accounted for by measurements of the Co^{58}-cyanocobalamin in liver, kidney, the remaining carcass, and

the urine. This percentage was lower in the HFLC-dieted animals, perhaps because of the greater technical difficulties of urine collection from the animals that had low urinary output. Because of this, the figures for a low urinary output of Co^{58}-cyanocobalamin in the animals on the HFLC diet are uncertain.

DISCUSSION

The problem of distribution of Co^{60}-cyanocobalamin in the normal rat has been studied extensively.[4-7] The concentration of the labeled vitamin in the liver increases gradually within 2 to 3 weeks following injection, and then reaches a plateau which is maintained for several weeks. In the kidney, however, the maximal concentration occurs on the second to fourth day following injection and declines rapidly. The rat belongs to a group of animals which has a higher content of vitamin B_{12} in the kidney than in the liver.

The difference in the Co^{58}-cyanocobalamin content between the liver of the malnourished animals 2 days and 2 weeks following the injection seems to indicate that the fatty liver loses the previously present Co^{58}-cyanocobalamin. This may occur because of actual diminution of the number of cells, i.e., cell necrosis. If this were the sole cause, the content of Co^{58}-cyanocobalamin per gram of fat-free liver tissue should not markedly differ from the normal liver. This was the case if the measurements were made 2 days following the injection, but 2 weeks following the injection, the obtained values differed by more than a factor of 2 (Table I). This appears to indicate an actual defect in the cyanocobalamin-binding capacity of the parenchymal cells of the animal on the hypolipotropic diet. It deserves emphasis that at the end of 40 days, no increased fibrosis was noted. Thus, it may be assumed that the weight of the liver following deduction of fat represented mainly cells. The diminution of the Co^{58}-cyanocobalamin concentration in the fully developed cirrhotic liver was not as marked as in the animals with fatty metamorphosis.

The reciprocal relationship of the vitamin B_{12} binding capacity of the liver and kidney of the rat is an interesting phenomenon, the physiologic significance of which is a matter of speculation. It is possible that the binding capacity of the kidney remains latent and incompletely saturated when the binding capacity of the liver is normal. This additional binding capacity in the kidney may be utilized only when the liver is diseased and its storage capacity diminished. This also could partially explain the diminished Co^{58}-cyanocobalamin urinary excretion of the malnourished animals. It is also possible that biochemical alterations

TABLE III. RECOVERY OF CO^{58}-VITAMIN B_{12} FROM THE RATS TWO DAYS FOLLOWING INJECTION

		LIVER			KIDNEY				
	NUMBER OF ANIMALS	PER CENT DOSE	MμG PER GRAM	MμG PER GRAM FAT FREE	PER CENT DOSE	MμG PER GRAM	CARCASS PER CENT DOSE	48 HOUR URINE PER CENT DOSE	TOTAL RECOVERY PER CENT DOSE
Dieted	9	2.16 ± 0.68	5.02 ± 1.9	5.68 ± 2.1	19.00 ± 1.0	221 ± 21	18.78 ± 2.1	37.60 ± 14.0	77.54 ± 14.0
Control	7	1.69 ± 0.44	7.88 ± 2.4	8.26 ± 2.6	13.70 ± 2.8	280 ± 53	17.18 ± 5.4	62.70 ± 6.5	95.27 ± 8.9

The data are expressed by means (± standard deviation).

in the animal receiving the hypolipotropic diet leads to the higher concentration of vitamin B_{12} in the kidney. It should be emphasized that there were no gross histologic abnormalities present in the kidney of these rats. The increased Co^{58}-B_{12} content of the kidneys of the dieted animals is not explained by a generalized retention of the vitamin. The recovery of Co^{58}-B_{12} from the carcass was not significantly different in the 2 groups.

The low urinary excretion of Co^{58}-cyanocobalamin in the cirrhotic rat is also of interest because of the confusing nature of the available data on vitamin B_{12} excretion in the cirrhotic patient. While Jones, Mills, and Capps[3] found that the urinary excretion of vitamin B_{12} is increased (measured by microbiologic assay), Baker and his associates[9] reported that the cirrhotic patient is unable to excrete a loading dose of vitamin B_{12} as well as the normal subject. The significance of these apparently divergent results is not clear. Unfortunately, it is not known whether the human kidney resembles the kidney of the rat with respect to the storage of the vitamin. By surface counting, over the left kidney, Meyer and his associates[10] found high activity over the kidney, but it was still lower than over the liver. There were such wide variations in the normal and diseased subjects that the establishment of a definite pattern in disease was impossible.

SUMMARY

1. The distribution pattern of Co^{58}-cyanocobalamin, mainly in kidney and liver, has been studied in the normal rat and the rat with fatty metamorphosis and cirrhosis of the liver.

2. The fatty and cirrhotic liver has been found to have a diminished binding capacity for Co^{58}-cyanocobalamin. The data suggest that this binding capacity is more severely impaired in the fatty than in the cirrhotic liver.

3. In the normal rat, the concentration of administered Co^{58}-cyanocobalamin in the liver increases gradually and reaches a maximum around the fourteenth day; in the fatty liver the concentration is smaller on the fourteenth than on the second day.

4. The kidney of the animals with fatty metamorphosis and cirrhosis of the liver has been shown to have an increased binding capacity for Co^{58}-cyanocobalamin. In the normal kidney, the Co^{58}-cyanocobalamin concentration diminishes between the second and fourteenth days following the administration of the labeled vitamin. In contrast, the kidney of the animal with fatty metamorphosis of the liver maintains or increases its Co^{58}-cyanocobalamin content during this period.

5. It is suggested that the diminished binding capacity of the damaged liver leads to the utilization of other potential binding sites for the vitamin, especially the kidney.

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A STUDY OF CARBOHYDRATES IN HUMAN GALL BLADDER BILE

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HUMAN gall bladder bile contains a macromolecular complex of protein and bilirubin which is capable of binding considerable quantities of cholesterol in water solution.[1] Such a complex may be of importance in the prevention of gallstones. The mucoid nature of gall bladder bile suggests that carbohydrate may be an important component, and furthermore, histochemical studies indicate that the mucous membrane of the gall bladder does indeed secrete a carbohydrate-containing protein.[2] These studies were undertaken to: (1) quantitatively investigate the carbohydrate moity of gall bladder bile; (2) identify some of the carbohydrate components in bile; and (3) correlate such carbohydrates with the presence of gallstones.

MATERIALS AND METHODS

Human gall bladder contents were obtained by means of surgical procedures or by postmortem examination. Analyses were made of 28 normal gall bladder biles, of which 23 were obtained by postmortem and 5 by aspiration during operation. Twenty-two biles from gall bladders with stones were analyzed, of which 4 were obtained by postmortem and 18 by surgical procedures. These specimens were preserved at −15° C. until analyzed.

Chemical Methods.—Hexosamine concentrations were determined by the Boas modification of the Elson-Morgan reaction.[3] Optimum hydrolytic conditions with HCl were determined by varying the HCl concentration from 1 N to 6 N and the time from 1 to 24 hours. Maximum yield of hexosamine was obtained by hydrolyzing with 2 N HCl for 2 hours in a sealed glass tube at 100° C. The hydrolysate was passed through a Dowex 50 (−400 mesh) ion exchange column, washed with water, and the hexosamine eluted with 2 N HCl. Hydrolysis with Dowex 50 ion exchange resin as described by Paulson and Deatherage[4] under various conditions of acid concentration and time did not give as high a yield as that obtained with 2 N HCl.

Neutral sugar concentrations were determined by hydrolyzing with 2 N HCl for 2 hours in a sealed glass tube at 100° C., separation of the neutral sugars in the water wash from the Dowex 50 ion exchange column as used above, and analysis by the Anthrone procedure.[5] The optimum hydrolytic conditions were identical with those for hexosamines.

Nitrogen concentrations were measured by the micro-Kjeldahl method.[6] Protein nitrogen and nonprotein nitrogen concentrations were determined in the precipitate and supernatant after fractionation by sodium tungstate, by zinc sulfate, by 10 per cent trichloroacetic acid, and by 95 per cent ethanol.[7] Total nitrogen concentrations of the gall bladder fluid were also measured directly. Precipitable hexosamine and neutral sugar concentrations were determined after simple fractionation by the above methods.

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Hexosamines were tentatively identified by ion exchange chromatography on a 35 × 0.9 cm. Dowex 50 (–400 mesh) ion exchange column,[8] eluting with 0.3 N HCl. Neutral sugars were paper chromatographed in 3 solvent systems[9]: (1) butanol:ethanol:water (3:2:1) descending on Whatman No. 54, (2) ethyl acetate:acetic acid:water (3:1:3) ascending, and (3) butanol:pyridine:water (6:4:3) ascending on Whatman No. 1. The papers were developed by either ammoniacal silver nitrate or aniline phthalate. Hexuronic acid concentrations were estimated by the naphthoresorcinol method,[10] and bilirubin by the Malloy and Evelyn method.[11]

RESULTS

The concentrations of hexosamine, hexose, and nitrogen were significantly lower in the stone group as compared to the normal group (Tables I and II). Biles No. 23 and No. 25 in the stone group (Table II), which were somewhat elevated in concentration of these carbohydrates, were from patients with cystic duct obstruction and hydrops of the gall bladder. These biles, in distinction from normal biles with comparable carbohydrate levels, lacked viscosity, suggesting that the carbohydrate complexes were of a different nature. The ratio of hexosamine to hexose concentration did not vary significantly between the normal and abnormal groups and indicated that there was 1 mole of hexosamine to 3 of hexose. The ratio of hexosamine to total nitrogen concentration also

TABLE I. ANALYTICAL RESULTS OF BILES FROM NORMAL GALL BLADDERS

BILE NUMBER	AGE	RACE OR NATIONALITY	SEX	HEXOSAMINE (MG. % ± SE)	HEXOSE (MG. % ± SE)	TOTAL NITROGEN (MG. %)	BILIRUBIN (MG. %)	URONIC ACID (MG. %)
1				131 ± 3	390 ± 15	830		
2				192 ± 3	505 ± 25	1,100		
3				165 ± 7	446 ± 1	790		
5				247 ± 5	600 ± 20	1,390	1,420	360
6				159 ± 3	463 ± 1	1,260	454	260
8	50	N	F	132 ± 2	625 ± 10	775		
9	59	LA*	M	230 ± 1	650 ± 1	732		
10	70	W	M	117 ± 1	400 ± 5	866		
11	48	W	F	64 ± 3	437 ± 1	764	1,968	620
12	46	W	F	78 ± 2	440 ± 3	554	1,440	1,010
16				100 ± 1	235 ± 2			
21	46	N	F	174 ± 2	540 ± 1	2,360		755
22	78	W	F	64 ± 1	162 ± 1	153		
26	28	W	M	118 ± 4	337 ± 1	412		
27	31	W	M	87 ± 4	288 ± 12	2		
30	41	N	M	150 ± 3	285 ± 5		8	58
33	63	W	M	43 ± 1	195 ± 2		96	28
36	8	W	F	136 ± 2	647 ± 7			
37	52	N	M	148 ± 8	875 ± 10		656	575
38	30	W	M	19 ± 1	198 ± 1			
39†	60	W	M	138 ± 8	400 ± 30		696	380
40	36	W	F	466 ± 10	1,097 ± 1			
29†	65	N	F	1,000 ± 10	2,500 ± 10	1,032		
34	42	W	F	370 ± 2	1,200 ± 2		188	370
35	1	N	F	44 ± 5	155 ± 1		408	160
42†				69 ± 1	122 ± 2	150		
44†	60	W	M	131 ± 4	168 ± 4	73		
55†	82	N	F	312 ± 1	390 ± 1			
Mean ± SE				182 ± 35‡	526 ± 89‡	828		

Ratio hexosamine/hexose = 0.34. Hexosamine/N = 0.22.
*Latin American.
†Obtained at operation.
‡These compared with stone biles are accompanied by a probability p = < .0001.

TABLE II. ANALYTICAL RESULTS OF BILES FROM DISEASED GALL BLADDERS

BILE NUMBER	AGE	RACE OR NATIONALITY	SEX	TYPE OF STONE	HEXOSAMINE (MG. % ±SE)	HEXOSE (MG. % ±SE)	TOTAL NITROGEN (MG. %)	BILIRUBIN (MG. %)	URONIC ACID (MG. %)
4*				Pigment	47 ± 1	113 ± 1	430	280	107
7*	86	N	M	Pigment	33 ± 1	110 ± 15	224		
13	69	W	F	Pigment	11 ± 1	25 ± 5	115		
14	68	N	F	Pigment	18 ± 1	60 ± 8	229	0	105
17*	76	LA†	F	Cholesterol	21 ± 3	78 ± 8	49	0	0
18	28	N	F	Cholesterol	42 ± 3	103 ± 1	372		158
19	36	W	F	Mixed	116 ± 4	370 ± 8	559		
20	53	W	F	Mixed	11 ± 1	50 ± 15	130		
23	25	N	F	Mixed	215 ± 3	396 ± 1	832	0.1	15
24	43	W	F	Mixed	31 ± 1	92 ± 1	482		
25	41	N	F	Mixed	172 ± 1	284 ± 4	552	0	0
28	15	N	M	Pigment	34 ± 1	32 ± 1			
31	60	W	M	Mixed	39 ± 1	164 ± 10		0	0
32	22	W	F	Mixed	37 ± 5	150 ± 10			
41	61	W	M	Mixed	23 ± 4	305 ± 9			
46*	24	N	F	Cholesterol	47 ± 3	145 ± 4	375		
47	55	W	F	Mixed	24 ± 3	85 ± 5	644		
48	48	W	F	Cholesterol	19 ± 1	115 ± 2	259		
49	32	W	F	Mixed	69 ± 4	132 ± 1	653		
50	65	N	F	Mixed	44 ± 1	397 ± 4			
51	70	W	F	Mixed	53 ± 1	269 ± 3	613		
53	29	W	F	Cholesterol	30 ± 1	80 ± 1	542		
		Mean ±SE			52 ± 11‡	166 ± 28‡	415		

Ratio hexosamine/hexose = 0.31. Hexosamine/N = 0.12.
*Portmortem specimens.
†Latin American.
‡These, compared with normal biles, are accompanied by a probability p = < .0001.

appeared to be fairly constant among the 2 groups. Analysis of several biles obtained from the common bile duct revealed a mean hexosamine concentration ± SE of 12 ± 7 mg. per cent, significantly lower than that observed in gall bladder biles.

An analysis of the age, sex, and race differences revealed a larger percentage of females in the stone group. Examination of the data, however, revealed that sex distributions could not explain these analytic differences. Furthermore, on comparing the data from surgical and autopsy specimens in each group, it did not seem likely that the differences could be the result of postmortem changes.

TABLE III. HEXOSAMINE CHROMATOGRAPHY

NORMAL BILES		STONE BILES	
BILE NUMBER	GLUCOSAMINE: GALACTOSAMINE	BILE NUMBER	GLUCOSAMINE: GALACTOSAMINE
11	5.4	17	4.1
21	2.6	18	2.8
26	2.5	23	2.0
29	1.4	23	2.7
29	2.5	25	3.7
36	3.1		3.07
37	4.1		
40	4.4		
	3.27		

Preliminary identification of the hexosamines by ion exchanges chromatography revealed that both glucosamine and galactosamine were present in a ratio of approximately 3 to 1 in both the normal and stone groups (Table III, Fig. 1).

Paper chromatography of the neutral sugars suggests the presence of relatively large amounts of galactose and smaller amounts of glucose, arabinose, fucose, and ribose (Figs. 2 and 3). Hexuronic acid, probably glucuronic, seemed also to be present on these chromatograms. Chromatography before and after dialysis revealed that the major dialyzable hexose was glucose.

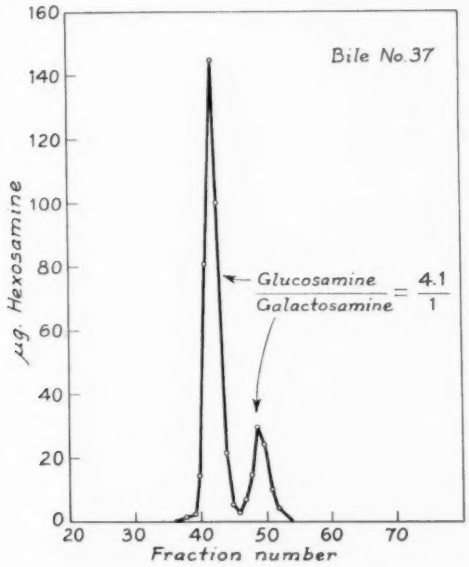

Fig. 1.—Chromatogram for hexosamines. Chromatographed on Dowex 50 (−400 mesh) 0.9 × 35 cm. column. Each fraction represents 1.5 ml. of eluent.

Hexuronic acid concentrations in a number of biles from both groups did not correlate with the hexosamine, hexose, or nitrogen concentrations, suggesting that the hexuronic acid was not part of the carbohydrate complex. There was, however, a fairly good correlation between hexuronic acid and bilirubin concentrations in these biles. Chromatography of several biles before and after dialysis revealed that the major portion of the uronic acid was nondialyzable (see Fig. 3). The amount of hexuronic acid dialyzable in 3 normal biles ranged from 30 to 60 per cent, averaging 40 per cent.

Preliminary observations of some of the physical and chemical properties of these carbohydrates in bile revealed that negligible amounts of hexosamine, 20 per cent of the hexoses, and 40 per cent of the nitrogen were dialyzable (Table IV). Ethanol, sodium tungstate, zinc sulfate, and trichloroacetic acid precipitated widely varying amounts of hexosamine and nitrogen, which suggests that the carbohydrate moity and the major portion of the nitrogen or protein are only loosely bound if at all.

TABLE IV. PRECIPITABLE NITROGEN AND HEXOSAMINE OF SIX NORMAL BILES

METHOD	% NITROGEN PRECIPITABLE	% HEXOSAMINE PRECIPITABLE	% HEXOSE PRECIPITABLE
Zinc sulfate	81	48	
Sodium tungstate	38	14	
10% trichloroacetic acid	49	57	
95% ethanol (10)*	38	91	74
	% nitrogen nondialyzable	% hexosamine nondialyzable	% hexose nondialyzable
Dialysis (2)*	61	96	80

*The numbers in parentheses indicate the number of biles studied.

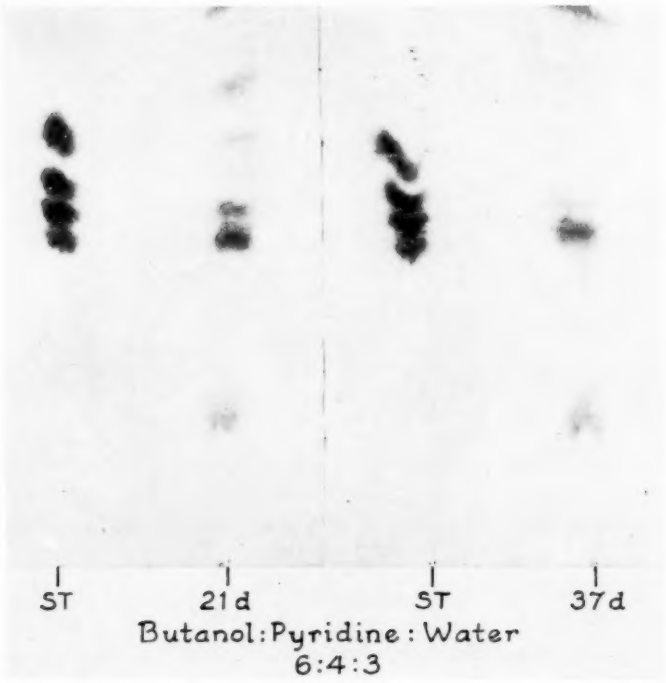

Fig. 2.—Chromatogram for neutral sugars. Whatman No. 1 paper with 3 ascending developments, stained with ammoniacal silver nitrate. The spots reading from bottom to top are: *ST*, standard galatose, glucose, arabinose and mannose (one spot), fucose and ribose (one spot); *21d* and *37d*, relatively large amounts of galatose with small amounts of glucose can be identified. The samples labeled *d* were dialyzed.

The quantitative importance of these carbohydrates in bile is emphasized by the observation that in one bile, No. 29, hexosamine constituted 4 per cent, and neutral sugars 11 per cent, or a total of 15 per cent of the total dry weight (Table V). Furthermore, if one assumes that all the nondialyzable nitrogen represents protein, normal biles have a mean protein content of 3.1 Gm. per cent of which the total carbohydrates, excluding hexuronic acid, represents 18 per cent of the sum of the protein and carbohydrate.

Histologic observations of the mucosa revealed that in general when the hexosamine concentration of the gall bladder fluid was low, the mucous membrane was denuded or markedly diseased as would be expected were the carbohydrates

TABLE V. RELATIVE AMOUNTS OF CARBOHYDRATE IN GALL BLADDER BILE

Bile No. 29: hexosamine + hexose = *15%* of total dry weight	
Normal biles: Mean (total protein*)	3.10 Gm. %
Mean (hexosamine + hexose)	.70 Gm. %
Total	3.80 Gm. %
Hexosamine + hexose = *18%* of Total	

*Calculated from mean nondialyzable nitrogen (60% of 828 mg. % × 6.25).

secreted by the mucous membrane. One bile, No. 53, however, obtained from a gall bladder with histologically normal mucous membrane had a low hexosamine concentration and a solitary stone.

Finally, analyses of several stones of both pigment and cholesterol types revealed only small amounts of hexosamine or neutral sugar. Five cholesterol

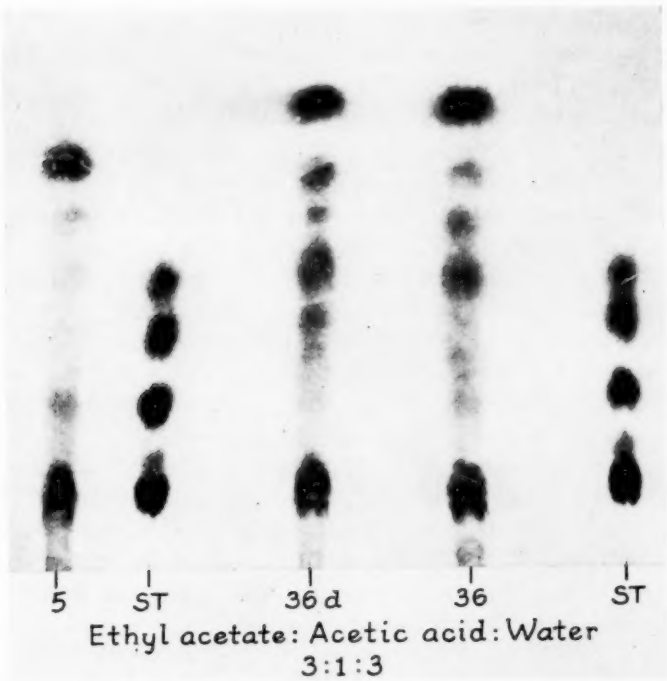

Fig. 3.—Chromatogram for neutral sugars. Whatman No. 1 paper with 4 ascending developments, stained with ammoniacal silver nitrate. The spots reading from bottom to top are: *ST*, galactose and glucose (one spot), mannose, arabinose, fucose, ribose; *5*, *36d*, and *36*, arabinose, fucose, and ribose may be identified. The uppermost spot in *36d* and *36* has the same Rf as glucuronolactone. The samples labeled *d* were dialyzed.

stones had a mean hexosamine concentration of 0.013 Gm. per cent dry weight while 3 pigment stones had a mean hexosamine concentration of 0.42 Gm. per cent dry weight.

DISCUSSION

Although the gall bladder mucous membrane may increase the viscosity of bile by concentrating processes, it also apparently secretes substances which may contribute to the viscosity. Verschure[1] has demonstrated that a lipoprotein is secreted by the gall bladder, while other investigators[2] have demonstrated by histochemical methods that carbohydrate substances are also secreted.

The lipoprotein seems to be of importance in stabilizing bile colloids, a role which the carbohydrate substances, if part of the lipoprotein complex, may also play. The possibility, however, that a mucoprotein may be a causative factor in gallstone formation has a very sound analogy in kidney stone formation, because a urinary glycoprotein containing hexosamine apparently forms the nidus for kidney stones.[12]

The observations presented here verify the histochemical evidence for carbohydrates in gall bladder bile, and furthermore emphasize the quantitative importance of these substances. These carbohydrates, exclusive of uronic acid, may constitute more than 15 per cent of the total solids of gall bladder bile.

The difference between the carbohydrate concentrations of biles from normal and pathologic gall bladders is significant statistically. The reduced concentrations of carbohydrate observed in biles from pathologic gall bladders may be only the result of destruction or disease of the mucous membrane, although at least 1 bile with a low hexosamine concentration and a stone was obtained from a gall bladder that was histologically normal. Whether such reduced concentrations play any role in the pathogenesis of gallstones has not been established.

Several of the carbohydrates present in gall bladder bile have been tentatively identified by chromatographic procedures. Both glucosamine and galactosamine are present in ratios of 3 to 1. No difference in this ratio was noted among the normal or pathologic biles. The predominant neutral sugar was galactose with smaller amounts of glucose, arabinose, fucose, and ribose. There were approximately 3 moles of these neutral sugars to 1 mole of hexosamine in both the normal and pathologic biles. The correlation between the concentrations of these neutral sugars and hexosamines and some of their chemical properties suggests that they are bound together as a carbohydrate complex, probably with a small amount of protein. The relative behavior of these carbohydrates and the nitrogen with various protein-precipitating agents indicates that the greater part of the bile proteins are not part of the carbohydrate complex. Further studies are necessary to establish how much the biliary proteins are involved in this carbohydrate complex.

SUMMARY

A carbohydrate complex found in human gall bladder bile contains predominantly glucosamine, galactosamine, and galactose with smaller amounts of glucose, arabinose, fucose, and ribose, and probably also contains some protein. This complex is apparently secreted by the gall bladder mucosa. It appears to be diminished in quantity in most gall bladders containing stones. Decreased concentrations of this complex may be merely a result of diseased gall bladder mucous membrane; but whether this complex contributes in any way to the stability of saturated gall bladder bile or has any role in the prevention, or cause, of gallstones, has not been established.

We are indebted to Drs. Ben Wilson and G. T. Shires and other members of the Departments of Surgery and Pathology for procuring many of these specimens.

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LABELING OF ANTIBODY AGAINST THE EHRLICH MOUSE ASCITES CARCINOMA WITH TRITIUM (H^3)

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RECENT developments in counting and labeling techniques have resulted in a considerable increase of interest in the potentialities of tritium (H^3) as a biologic label. Chen[1] and also Vaughn, Steinberg, and Logan[2] have investigated several problems associated with liquid scintillation counting of this isotope in biologic fluids and compounds. A major impetus in the field has been the simple method of tritium labeling developed by Wilzbach,[3] which has been applied to a number of biologic materials, including enzymes,[4] and which results in a relatively random exchange with labile hydrogens of the molecule undergoing labeling. After careful purification, tritiated compounds of relatively high specific activity are obtained.

The successful labeling of lysozyme and ribonuclease[5] by this method suggests that complex biologic molecules of other kinds, for example, antibodies, may be labeled in this manner. Antibodies labeled with tritium may be effectively utilized in several areas of immunology, particularly those involving determination of localization in vivo of antigens and antibodies. Promise of utility in the latter area depends, in part, on the remarkable suitability of tritium as a label in radioautographic work, because of the short track of the β-radiation of this isotope on photographic emulsions. This report describes the preparation of tritiated tumor antibody by the Wilzbach method, and a study of some of the properties of the labeled antibody.

MATERIALS AND METHODS

Preparation and Fractionation of Immune Serum.—Seven rabbits were immunized, using progressively increasing doses of washed suspension of Ehrlich mouse ascites tumor cells. Details of collection and washing of tumor cells, together with details of the immunization method, have been reported previously.[6] Pooled antisera were fractionated by the cold-ethanol procedure of Deutsch[7] to yield the γ_2-globulin fraction, which was dialyzed against distilled water and stored in the lyophilized form. Unless further purified, γ_2-globulin prepared in this way carried appreciable contamination due to varying amounts of material resembling γ_1-globulin. The total fraction will be referred to as whole γ_2-globulin.

Tritiation of Whole γ_2-Globulin.—One hundred milligrams of the lyophilized material was dissolved in 20 ml. of 0.85 per cent sodium chloride solution, and centrifuged to remove traces of insoluble material. The solution was exposed, under reduced pressure, to the action of 2 to 5 curies of carrier-free tritium gas* for 48 to 72 hours at 5° C. After exposure, the

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tritiated sample was lyophilized to remove tritiated water and labile tritium. The dry material was dissolved in water, and dialyzed for 48 hours against distilled water. Insoluble material was removed by centrifugation, and the solution was lyophilized.

Anion Exchange Chromatography.—Labeled and unlabeled whole globulin samples were characterized by gradient elution chromatography, after the method of Sober, Gutter, Wyckoff, and Peterson,[8] using diethylaminoethyl (DEAE) cellulose (Brown Company, Berlin, New Hampshire). A 2.5 by 20 cm. cellulose column, equilibrated with 0.005 M (pH 7.0) phosphate buffer, was used. Aqueous globulin samples of 75 to 100 mg. were dialyzed against this buffer for 24 to 48 hours, and applied to the column in a 10 ml. volume. Gradient elution was carried out in a 200 ml. mixing chamber. The following series of eluants were used: (1) 0.005 M phosphate buffer, pH 7.0, 125 ml.; (2) 0.02 M phosphate buffer, pH 6.0, 200 ml.; (3) 0.05 M monobasic sodium phosphate, pH 4.6, 250 ml.; (4) 0.05 M monobasic sodium phosphate-sodium chloride, pH 4.6, 250 ml.; and (5) 1 M (approximately) sodium chloride, 200 ml. The average flow rate was 20 ml. per hour.

Estimation of Total Protein in Chromatography Eluates.—Protein concentration was estimated by ultraviolet absorption spectrophotometry at 280 mμ. Results were intermittently checked by a modification of the colorimetric method of Folin.[9]

Electrophoretic Characterization.—The composition of whole labeled and unlabeled globulins, and of chromatography eluates, was examined by paper strip electrophoresis. Samples were dissolved in, or dialyzed against, 0.85 per cent sodium chloride solution before use at an approximate concentration of 10 mg. per ml. In the case of chromatography eluates, concentration to this value was performed by dialysis against 15 per cent polyvinylpyrrolidone. Ten lambda portions were applied to individual paper strips (S and S 470) with a wire loop applicator, and electrophoresis was carried out in a Durrum type electrophoresis cell (Spinco Model R, Series D) in Veronal buffer (pH 8.6, ionic strength 0.075), at room temperature. A current of 20 milliamperes was applied for 18 hours, the strips dried at 120° C., and were stained with 0.1 per cent bromophenol blue in methanol; excess stain was removed with 5 per cent acetic acid, and the color was developed with ammonia. Electrophoretic patterns were recorded with an automatic integrator-densitometer (Spinco Analytrol, Model RB).

Ultracentrifugal Characterization.—The composition of whole labeled and unlabeled globulins was examined by ultracentrifugation in a Spinco ultracentrifuge (Model E). Samples were centrifuged at 59,780 r.p.m. at 20° C., at an approximate concentration of 10 mg. per ml. (in 0.15 M sodium chloride), for 90 minutes.

Estimation of Combining Activity.—Combining activity was measured by the quantitative (50 per cent) complement fixation test,[10] using washed suspensions of Ehrlich mouse ascites carcinoma cells as the test antigen. Hemolysis was measured spectrophotometrically. Combining activity was determined for untritiated whole globulin, tritiated whole globulin, and peak 5 (sodium chloride) eluates by the customary doubling dilution method of assay, and the end point expressed as the highest dilution exhibiting a signficantly lower optical density than the (50 per cent hemolysis) control. The whole globulins were dissolved for assay at a concentration of 10 mg. per ml., in 0.85 per cent sodium chloride solution, containing 0.20 per cent magnesium sulfate (Mg saline). The peak 5 eluates were concentrated down to 10 mg. per ml. and then dialyzed against Mg saline, at constant volume, before use. Samples from peaks 1, 2, 3, and 4 did not contain sufficient protein to permit concentration to 10 mg. per ml., and thus could not be assayed by the doubling dilution method used above. Instead, samples from these peaks were dialyzed, at constant volume, against Mg saline, and 0.2 ml. of each was used without concentration and without serial dilution, in a single-tube assay. The other reagents were the same as in the serial dilution test. After lysis, the optical density of the supernate was compared with optical density values on a standard hemolysis curve ranging from 0 to 50 per cent hemolysis, and finally expressed as per cent hemolysis. Since the control (no antibody added) was adjusted to result in 50 per cent hemolysis, the latter value,

if obtained with an eluate sample, indicated an absence of antibody activity in that sample. It should be noted that these per cent hemolysis values are not directly comparable to the results of serial dilution tests. In addition, the relative antibody content of peaks 1, 2, 3, and 4 cannot be directly compared, the per cent hemolysis values serving essentially as indicators of the presence or absence of combining activity.

In Vivo Half-Life of Tritiated Globulin.—Three male rabbits, of similar body weight, were intravenously injected with 15 mg. whole tritiated globulin in a volume of 1.0 ml. After a 15 minute equilibration period, 2 ml. blood samples were withdrawn from each animal; samples were also taken every day for 5 days following administration of labeled globulin, and also on the eighth and twelfth days. Samples were allowed to clot, and the centrifuged serum was used for estimation of residual tritium radioactivity. As a partial control on the rate of removal of tritiated water (formed by exchange with the labeled globulin administered), 1.0 ml. of tritiated water was injected intravenously into rabbits, and residual tritium radioactivity was estimated over a 4 day period, as before, except that 0.5 ml. of whole blood was withdrawn for counting.

Estimation of Tritium Radioactivity.—In the case of whole labeled globulin, samples were weighed into counting vials, and 1.9 ml. of Hyamine solution (hydroxide form, in methanol-toluene)[11] added. When serum was to be counted, 0.2 ml. portions of serum were placed in counting vials, and dried in vacuo over phosphorus pentoxide for 48 hours, after which Hyamine solution was added. Suspensions in Hyamine solution were incubated in the sealed vials, overnight at 40° C. to 45° C., after which time all solid material dissolved. Nine milliliters of a toluene solution of phosphor and spectrum shifter[12] was added, the contents mixed, and counts per minute counted on a Packard Tri-Carb liquid scintillation counter. Quenching effects of Hyamine, and of protein in individual samples, were estimated by the use of standard tritiated toluene (New England Nuclear Corporation NES-4) and corrected

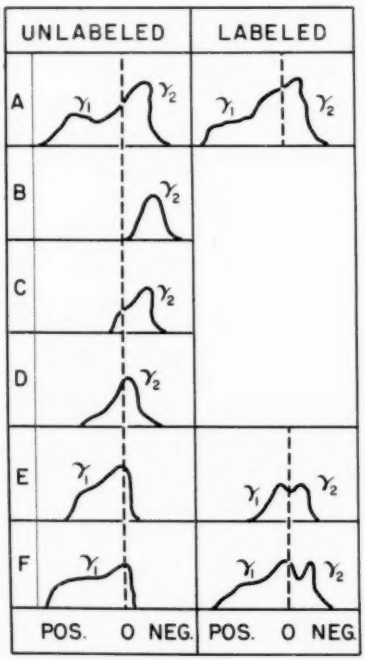

Fig. 1.—Paper electrophoresis patterns of whole (once fractionated) γ_2-globulin fractions, and of chromatography eluates, before and following tritiation. *A*, whole globulin; *B*, chromatography eluate, peak 1; *C*, peak 2; *D*, peak 3; *E*, peak 4; *F*, peak 5.

results expressed as disintegrations per minute. Specific activities were expressed as millicuries per gram protein. When chromatography eluates were to be counted, 0.5 ml. of aqueous sample was directly mixed with 1.0 ml. of Hyamine solution, and 3.0 ml. methanol, 5.5 ml. of phosphor-spectrum shifter were then added. Subsequent procedures were as before; corrections were made for the additional quenching effects of excess methanol.

RESULTS

Following tritiation, and subsequent lyophilization, the solid labeled globulin was partially insoluble in 0.85 per cent sodium chloride solution. The insoluble fraction amounted to between 10 and 15 per cent, by weight, of the original material. After removal of the insoluble fraction, and relyophilization, the resulting powder retained the ability to dissolve completely in dilute salt solution. Soluble fractions (whole labeled globulin) had specific activities of between 1 and 10 mc. per Gm., depending on the amount of radioactivity used, and the duration of the labeling procedure.

Whole unlabeled γ_2-globulin, as prepared by a single fractionation from immune serum, usually contained varying amounts of a material resembling γ_1-globulin, as evidenced by the results of paper electrophoresis (Fig. 1). For convenience, this material will be referred to as γ_1-globulin, although its solubility and electrophoretic mobility are intermediated between γ_2- and γ_1-globulin. Ultracentrifugation patterns also showed the presence of a major component,

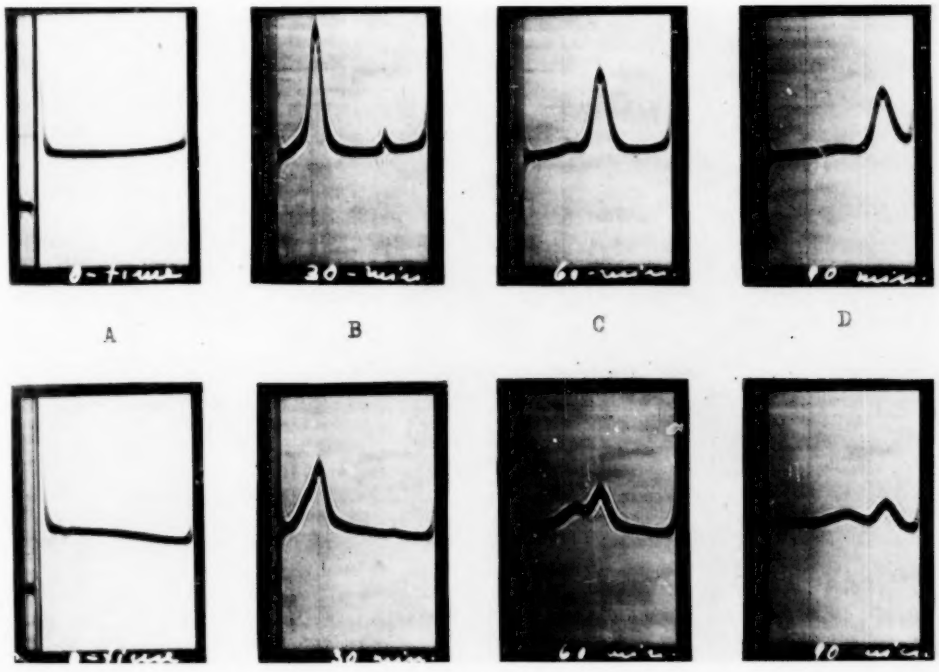

Fig. 2.—Ultracentrifuge patterns of whole (once fractionated) γ_2-globulin fractions, before and following tritiation. Spinco Model E, 20° C., 59,780 r.p.m. *A*, Zero time; *B*, 30 minutes; *C*, 60 minutes; *D*, 90 minutes; upper picture = unlabeled globulin; lower picture = labeled globulin.

with a faster moving minor component (Fig. 2). The combining activity of antibodies present in whole unlabeled globulin, when expressed as a doubling-dilution titer, was 1:1,280. The properties of whole labeled globulin were similar, but not identical. Complement fixation titers were unchanged, within the limits of this test, and paper electrophoresis yielded essentially the same results, indicating the presence of both γ_2- and γ_1-globulins (Fig. 1). Ultracentrifugation patterns, however, showed the existence of not only the original components, but also of a third, slower moving component (Fig. 2).

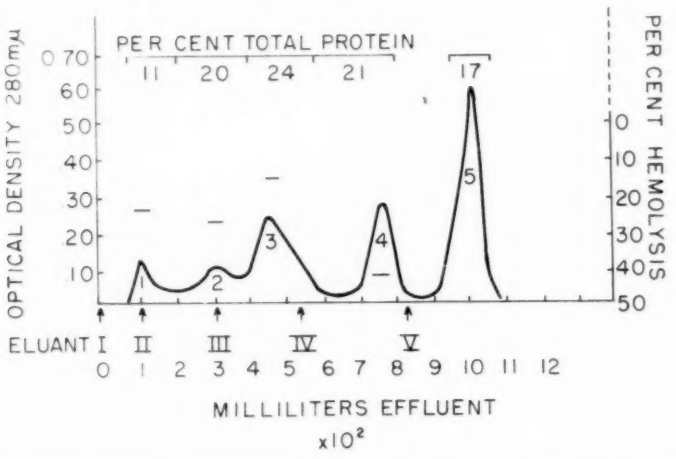
PER CENT TOTAL PROTEIN
11 20 24 21 17
OPTICAL DENSITY 280 mμ
0.70 60 50 40 30 20 10
PER CENT HEMOLYSIS
0 10 20 30 40 50
1 2 3 4 5
ELUANT I II III IV V
0 1 2 3 4 5 6 7 8 9 10 11 12
MILLILITERS EFFLUENT
x10²

Fig. 3.—Anion exchange chromatography: subfractionation of unlabeled whole γ_2-globulin. Gradient elution on DEAE cellulose. Roman numerals represent points of eluant application, peak numbers *1* through *5*, in order of emergence. Solid line refers to protein elution (O. D. 280 mμ); bars refer to combining activity (per cent hemolysis). Peak *5* gave no titer in a serial dilution complement fixation test.

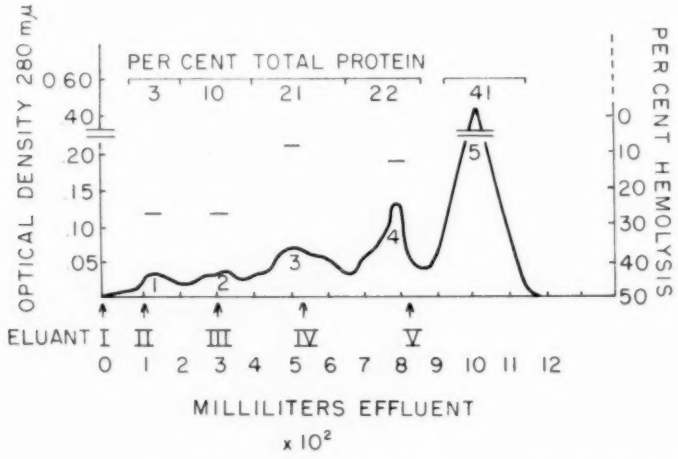
PER CENT TOTAL PROTEIN
3 10 21 22 41
OPTICAL DENSITY 280 mμ
0.60 40 20 15 10 05
PER CENT HEMOLYSIS
0 10 20 30 40 50
1 2 3 4 5
ELUANT I II III IV V
0 1 2 3 4 5 6 7 8 9 10 11 12
MILLILITERS EFFLUENT
x 10²

Fig. 4.—Anion exchange chromatography: subfractionation of labeled whole γ_2-globulin. Gradient elution on DEAE cellulose. Roman numerals represent points of eluant application, peak numbers *1* through *5*, in order of emergence. Solid line refers to protein elution (O. D. 280 mμ); bars refer to combining activity (per cent hemolysis). Peak *5* titered at 1:640 in a serial dilution complement fixation test.

Anion exchange chromatography of whole unlabeled globulin resulted in separation into 5 major peaks (Fig. 3). When combined eluates corresponding to the different peaks were characterized by paper electrophoresis, it was found that peaks 1, 2, and 3 contained γ_2-globulin only (11, 20, and 24 per cent, respectively, of original load), while peaks 4 and 5 contained γ_1-globulin only (21 and 17 per cent, respectively). γ_2-globulin fractions showed a gradual increase in electrophoretic mobility from peak 1 through peak 3 (Fig. 1). Antibody combining activity was present in eluates corresponding to peaks 1 and 2, and to a lesser extent, to peak 3; essentially no activity was present in eluates corresponding to peaks 4 and 5.

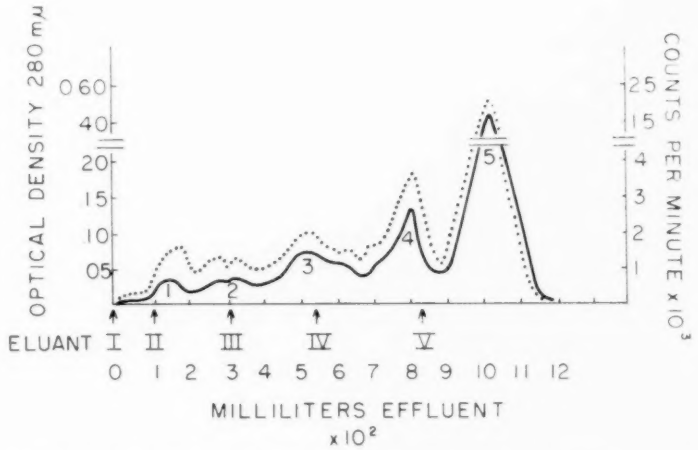

Fig. 5.—Subfractionation of whole labeled γ_2-globulin on DEAE cellulose: relative elution of protein and tritium radioactivity. Gradient elution. Roman numerals represent points of eluant application, peak numbers *1* through *5*, in order of emergence. Solid line refers to protein elution (O. D. 280 mμ). Broken line refers to elution of tritium radioactivity (c.p.m. times 10^3).

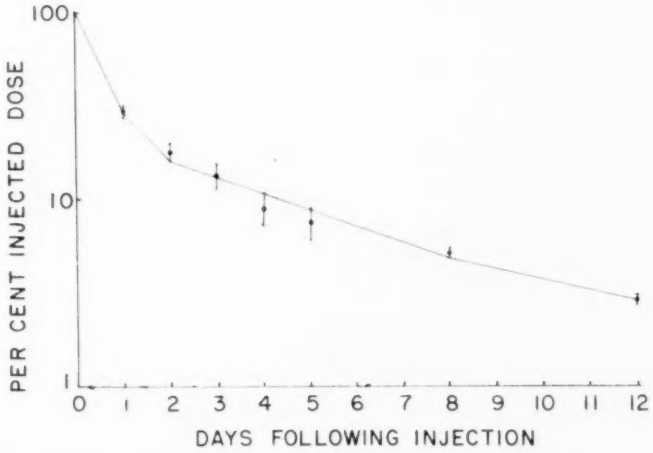

Fig. 6.—Elimination of tritiated rabbit globulin from rabbit blood. Average of points for 3 animals. Vertical lines represent standard deviations from the mean. Intravenous dose was 15 mg. whole tritiated globulin. Zero time was taken as 15 minutes following injection.

When whole labeled globulin was fractionated by anion exchange chromatography, certain striking changes were evident. Although 5 major peaks, corresponding closely to those obtained with unlabeled material, were obtained (Fig. 4), it was noted that a significantly smaller portion of the protein was recovered in the original peaks, and a correspondingly larger portion in peak 5 (3, 10, 21, 22, and 41 per cent, respectively, of the original protein load). Paper electrophoresis showed that peaks 4 and 5 now contained a mixture of γ_2- and γ_1-globulins (Fig. 1) and also contained materials exhibiting antibody activity. Thus peak 5 eluate had a complement fixation doubling-dilution titer of 1:640, at a protein concentration of 10 mg. per ml.

When counts per minute of tritium radioactivity of the eluate fractions were plotted, the radioactivity was found to coincide closely with the protein elution peaks, all peaks exhibiting the presence of the tritium label. No other radioactivity peaks were found (Fig. 5).

The elimination of whole labeled globulin from the blood of injected rabbits corresponded to the typical pattern expected of homologous protein. After a drop in radioactivity, due to initial equilibration, radioactivity was lost from the blood at a slow rate, with a half-life of approximately 3.4 days. By the twelfth day, no increase in the elimination rate, corresponding to immune response, had occurred (Fig. 6). When tritiated water alone was injected, tritium radioactivity disappeared rapidly from the circulation; only a trace of the injected radioactivity was detectable 15 minutes following injection.

DISCUSSION

The advantages of tritium labeled antibodies are readily apparent, and indeed, this material has already been prepared by biosynthesis.[13] Drawbacks of expense, yield, and low specific activity are inherent in the biosynthetic method, and warrant a study of simple in vitro labeling methods. The remarkably wide applicability of the method of gaseous exchange would seem to place this procedure in the latter category.

Experience with the Wilzbach method has shown that a certain amount of degraded, highly radioactive material usually appears following labeling[14]; thus, it is necessary to subject the crude product to careful purification. In the present instance, major emphasis may be placed on the removal of material with a diminished or changed biologic activity, and the retention and characterization of the remainder; ideally, the latter should have retained combining power, and, in the homologous animal, should be disposed of in the same manner as native protein.

Following labeling, a certain amount of frank denaturation was observed. After removal of insoluble protein, the remaining soluble material may be regarded as being composed of unchanged protein, together with variable amounts of species which have undergone varying degrees of molecular change; the latter may, or may not, have suffered concomitant changes in biologic activity. Ultracentrifugation findings indicated that a certain degree of alteration in physical properties had occurred, and this finding, together with the

change in composition of the peaks obtained by chromatography, presumably indicates an increase in molecular heterogeneity of the labeled material. It is notable that the shift in elution of γ_2-globulin was accompanied by a parallel shift in the distribution of combining power, suggesting that molecular changes were not sufficiently great as to be accompanied by major loss in biologic activity.

The close coincidence of the curve for tritium label distribution with the protein distribution curve, together with the absence of other radioactivity peaks, indicates that an essentially uniform distribution of label through the component proteins occurred; and that following removal of insoluble material, no fractions with a major concentration of label were present.

Whole labeled and unlabeled globulin fractions gave essentially similar doubling-dilution titers in the quantitative complement fixation test. The curve for disappearance of the former from the blood of the homologous animal species was almost identical with that described by Dixon, Bukantz, and Dammin,[15] for radioiodinated γ-globulin in the rabbit. The half-life of the tritiated material was 3.4 days, which agrees closely with the figure derived from the data presented in the report just mentioned. More recent values for the half-life of ethanol-fractionated rabbit γ-globulin in the rabbit are of the order of 4.6 days[16]; further experience with tritium labeling, for example, labeling in the solid state, may result in preparations with half-lives approaching the latter figure. No indication of an increase in the rate of clearance, corresponding to antibody produced by the rabbit, was observed over a 12 day period. The reliability of the half-life value obtained is subject to question, since the degree of exchange between labeled protein and serum water is not known. A partial control, consisting of intravenous administration of tritiated water to rabbits, indicated very rapid disappearance of radioactivity from the blood. If a similar disappearance rate of tritiated serum water occurs when labeled protein is injected, the half-life value obtained for the latter probably represents a minimum value.

SUMMARY

1. The γ_2-globulin fraction of rabbit antisera against the Ehrlich mouse ascites carcinoma was labeled with tritium by the method of gaseous exchange.

2. After removal of insoluble products, the tritiated material had increased in molecular heterogeneity, but had retained antibody titer, and was handled by the homologous animal in a manner similar to that encountered with native protein.

3. In the course of this work, rabbit γ_2-globulin (ethanol fractionated) has been characterized by anion-exchange chromatography.

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EFFECT OF NEOMYCIN ALTERATION OF THE RAT INTESTINAL FLORA ON SERUM CHOLESTEROL AND VALVULAR SUDANOPHILIA

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AHRENS[1] has recently re-emphasized the relationships among diet, the intestinal microflora, and intestinal absorption with respect to serum lipid levels and experimental atherogenesis.

Rats, raised in a germ free environment[2] or treated with chemotherapeutic agents,[2, 3] exhibited greatly decreased fecal excretion rates of labeled cholic acid. Oral administration of penicillin and Sulfasuxidine has been shown by Coleman and Baumann[4] to depress the fecal excretion of coprostanol with a corresponding increase in fecal cholesterol. Thus, the fecal cholate excretion in these studies appears to be governed by, or, at least, to be related to alterations in the intestinal flora.

Rosenfield[5] reported that dietary cholesterol can undergo direct conversion to coprostanol, a nonabsorbable sterol, within the intestinal lumen—presumably because of the activity of the microorganisms present.

Furthermore, Portman and associates[6] demonstrated elevation of serum cholesterol in rats which were maintained on a cholesterol-cholate starch diet plus Sulfasuxidine (as compared to the same diet without Sulfasuxidine). Presumably the difference in serum cholesterol level was related to alterations in the intestinal flora since it was not observed in the absence of the drug.

The present experiment was undertaken to study the effect of neomycin-altered intestinal flora upon the absorption of cholesterol, as evidenced by changes in serum cholesterol levels and aortic and heart valve sudanophilia.

Antibiotics administered orally have been reported to increase the absorption of various mineral elements, i.e., calcium[7] and manganese.[8] Since increased intakes of dietary magnesium have been shown to retard the development of vascular sudanophilia,[9] the effect of neomycin on the absorption of dietary magnesium was also included in this study.

METHODS

Weanling male albino rats, weighing 50 to 55 grams, were housed in individual cages and fed ad libitum on a basic diet containing purified casein (10 per cent), glucose (58.1

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per cent), salt mixture (5 per cent), Celluflor (5 per cent), choline chloride (0.3 per cent), vitamin ADE mixture (0.1 per cent), $CaCO_3$ (1.5 per cent), and fat (Spry) (20 per cent). Another group was fed this diet plus 1 per cent cholesterol and 0.3 per cent cholic acid. A constant percentage of protein and fat was maintained by reducing glucose proportionately. Animals on both diets were further divided into those receiving either water, or water containing neomycin sulfate* (a nonabsorbable antibiotic) in a concentration of 60 mg. per 100 ml. Magnesium in the form of magnesium oxide was added in concentrations of 6, 12, 24, 96, and 192 mg. per 100 Gm. diet.

Food and fluid intakes were measured daily. Animals were weighed twice weekly and observed for signs of magnesium deficiency, viz., hyperexcitability, and hyperemia of the ears, feet, and tail. As a check on the effect of the antibiotic regimen, fresh stool specimens were cultured at weekly intervals; coliform organisms were enumerated on MacConkey agar. Parallel total yeast counts were made on potato dextrose agar in order to ascertain the possibility of yeast superinfection. Plates were incubated at 37° C. for 48 hours.

After 28 days the animals were killed. The left kidneys were decapsulated and weighed; hearts were removed with the aorta attached so as to expose the aortic and mitral valves, were fixed in 10 per cent Formalin, and were stained with Sudan IV. Valvular and aortic sudanophilia was then estimated and the total vascular sudanophilia designated as "heart score" in the manner previously reported.[9] The degree of renal tubular microlithiasis was likewise evaluated and the "kidney score" was estimated, as an indication of magnesium deficiency, as has been previously described.[9] Serum cholesterol levels were determined by the method of Carpenter and associates.[10]

RESULTS

The addition of neomycin to the drinking water resulted in suppression of coliform organisms in the feces (Table I). The coliform count showed a marked drop within the first week and within 14 days fell from a normal level of approximately 8×10^9 to a level of less than 1×10^2 organisms per gram of wet feces. Resistant coliforms were not apparent nor was there any rise in yeast count throughout the period of this study.

Animals fed either the basic diet, or the cholesterol-cholate diet with or without neomycin, reached a maximal weight gain at a minimal dietary magnesium level of 12 mg. per cent (Tables II and III). Cholesterol-cholate-fed animals did not gain as much weight as did those fed the basic diet; neomycin-fed animals in all groups exhibited a greater weight gain than did those animals not fed neomycin.

Neomycin did not alter serum cholesterol levels of animals fed the basic diet. The serum cholesterol levels of animals fed the cholesterol-cholate diet averaged 650 mg. per cent, and the addition of neomycin resulted in a further increase of the average serum cholesterol levels to 1,062 mg. per cent, despite

TABLE I. EFFECT OF NEOMYCIN ON COLIFORM AND YEAST COUNT

NUMBER OF ANIMALS	NEOMYCIN	AVERAGE COUNT PER GRAM OF WET FECES (SEVENTH DAY)	(FOURTEENTH DAY)	(TWENTY FIRST DAY)
6	Coliforms	5×10^3	$< 1 \times 10^2$	$< 1 \times 10^2$
	Yeasts	3×10^5	3×10^6	3×10^5
6	Control			
	Coliforms	2×10^{10}	1×10^8	3×10^9
	Yeasts	2×10^5	3×10^7	8×10^9

*Mycifradin sulfate, The Upjohn Company, Kalamazoo, Mich.

TABLE II. THE EFFECT OF NEOMYCIN ON SERUM CHOLESTEROL, KIDNEY PROFILE, AND GROWTH OF RATS FED A BASIC DIET

DIETARY MAGNESIUM MG. PER 100 GM.	NEOMYCIN	SERUM CHOLESTEROL (MG. %)	KIDNEY PROFILE			WEIGHT GAIN (GM.)	AVERAGE CALORIC INTAKE PER DAY
			KIDNEY SCORE	KIDNEY WEIGHT (MG.)	KIDNEY : BODY WEIGHT RATIO		
6	− (6)*	82.0	3.4	622	.62	41	45.1
	+ (6)	84.0	3.6	780	.73	48	46.1
12	− (6)	90.0	2.5	679	.59	54	55.2
	+ (6)	98.3	3.0	681	.51	73	54.2
24	− (6)	90.0	1.3	490	.45	50	47.5
	+ (6)	88.3	1.1	601	.47	70	55.6
96	− (6)	76.0	0.3	476	.40	58	48.5
	+ (6)	93.0	0.1	566	.46	64	56.6
192	− (6)	75.0	0.1	482	.40	59	50.4
	+ (6)	84.3	0.02	577	.45	68	55.1

*Number of animals.

similar caloric intakes in both groups. Serum cholesterol levels were unaffected by dietary magnesium intake. Table III illustrates that the heart score of 5.3 was reduced to 1.9 in animals not fed neomycin, by increasing the magnesium from 6 to 96 mg. per cent. The addition of neomycin to the drinking water resulted in an over-all increase in heart score, at all levels of dietary magnesium. Increases in dietary magnesium reduced the heart score of 7.7 at 6 mg. per cent to 4.1 at 96 mg. per cent. However, the protective effect of magnesium on heart score was not altered by the neomycin.

The kidney:body weight ratio showed a progressive reduction with increasing dietary magnesium in all groups. It was not influenced significantly by any other factors.

On the other hand, the onset of hyperexcitability and peripheral hyperemia in neomycin-fed animals was delayed by at least one week in the four week experiment. In addition, daily observation of the degree of estimated hyperemia was significantly reduced by neomycin feeding.

TABLE III. THE EFFECT OF NEOMYCIN ON SERUM CHOLESTEROL, HEART SCORE, KIDNEY PROFILE, AND GROWTH OF RATS FED A CHOLESTEROL-CHOLATE DIET

DIETARY MAGNESIUM MG. PER 100 GM.	NEOMYCIN	SERUM CHOLESTEROL (MG. %)	HEART SCORE	KIDNEY PROFILE			WEIGHT GAIN (GM.)	AVERAGE CALORIC INTAKE PER DAY
				KIDNEY SCORE	KIDNEY WEIGHT (MG.)	KIDNEY : BODY WEIGHT RATIO		
6	− (4)*	762	5.3	3.3	572	.69	23	35.5
	+ (6)	1 089	7.7	2.4	531	.55	36	39.8
12	− (6)	645	5.6	3.3	624	.61	41	44.2
	+ (6)	1,043	6.4	1.5	522	.46	53	46.5
24	− (6)	601	3.2	1.2	463	.45	44	48.5
	+ (6)	818	6.9	1.5	504	.41	63	48.0
96	− (6)	596	1.9	0.7	462	.45	42	45.1
	+ (6)	1,136	4.1	0.5	489	.41	61	47.5
192	− (6)	653	2.1	0.4	447	.46	38	44.6
	+ (6)	1,233	4.9	0.3	484	.47	44	48.0

*Number of animals.

DISCUSSION

Addition of neomycin to the drinking water resulted in an increased weight gain in animals fed both diets. Further discussions concerning the well-known growth-promoting effect of antibiotics may be found in the excellent reviews of Jukes[11] and Porter.[12]

Neomycin administered orally did not affect the serum cholesterol levels of the rats fed a cholesterol-cholate free diet. Presumably, neomycin alteration of the intestinal flora impedes the degradation of bile acids in the hepato-biliary-intestinal circulation. This may be tantamount to increasing the level of absorbable cholic acid. However, in rats fed a basic diet with only cholic acid added, Beher and associates[13] observed a markedly decreased rate of in vivo hepatic cholesterol synthesis with a concomitant, but very slight, decrease in serum cholesterol. This may also partially explain the moderate lowering effect of oral neomycin on serum cholesterol levels in humans as reported by Samuel and Steiner.[14]

Animals fed a cholesterol-cholate diet exhibited an increase in the degree of hypercholesteremia when the diet was supplemented with neomycin. These observations lend support to the concept that a reduction of the intestinal flora impedes the degradation of the bile acids[4, 15] and cholesterol to nonabsorbable coprosterols,[3, 5, 16] and thus allows their reabsorption. Speculation cannot be ruled out that alterations in the intestinal flora, which are induced by antibiotics or specific dietary constituents, cause physical changes within the lumen of the intestine, which favor the absorption of a given nutritional substrate.

These findings are in agreement with those of Portman,[6] who obtained a moderate rise in serum cholesterol levels by the addition of Sulfasuxidine to the diet of cornstarch-cholesterol-cholate-fed rats.

Elevated levels of serum cholesterol were accompanied by increased deposition of sudanophilic material in the heart and aorta, but they were not directly proportional to one another. Cholesterol-cholate-neomycin-fed rats exhibited higher heart scores than did their neomycin-free counterparts. Vascular and aortic sudanophilia, however, was diminished by increasing amounts of dietary magnesium in both these groups, but was without effect on serum cholesterol levels.

Although the clinical symptoms of magnesium deficiency were lessened and body weight increased by feeding neomycin, the anatomic changes of the kidney, which were attributed to magnesium deficiency, were not improved by neomycin. Thus, it is not certain at this time that neomycin administered orally resulted in either increased absorption of magnesium or more efficient utilization. The techniques available for evaluation of magnesium deficiency used in this study were not sufficiently critical to evaluate these small differences.

SUMMARY

Alteration of the intestinal flora by neomycin feeding, as evidenced by decreased stool coliform counts of rats fed a cholesterol-cholate-free diet, had no

effect on serum cholesterol levels. However, rats fed diets containing cholesterol-cholate plus neomycin exhibited markedly higher serum cholesterol levels and an increased amount of vascular and aortic sudanophilia than did non-neomycin-fed counterparts.

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A FAMILY WITH HEMOGLOBIN G

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HEMOGLOBIN G was originally found by Edington and Lehman[1] in a West African family; it was characterized by a mobility between that of hemoglobin A and hemoglobin S in paper electrophoresis, Veronal buffer, pH 8.6. In free electrophoresis, cacodylate buffer pH 6.5, G is inseparable from S, but in free electrophoresis, Veronal buffer, pH 8.6, it is difficult to separate from A.[2] In chromatography on columns of ion exchange resin, G assumes a position between A and S.[3] The present report deals with a hemoglobin variant with these same characteristics, found together with hemoglobin A, in a Negro obstetric patient (E. T.) and each of her 5 children.*

MATERIALS AND METHODS

Hemoglobin solutions were obtained by centrifugation from erythrocytes washed with saline and hemolyzed by the addition of 1 volume water and ¼ volume toluene. Paper electrophoresis analyses, Veronal buffer, pH 8.6, μ 0.05, were made in the apparatus previously described.[4] In some instances, the filter paper used (Whatman No. 1) had previously been dipped into a 0.5 per cent solution of egg albumin and dried. Such papers minimized the "trailing" of hemoglobin and permitted sharper localization of the components. Paper electrophoresis with acid buffer (citrate, pH 6.5) was performed on Whatman No. 3 filter paper enclosed in glass plates held together with pressure clamps,[5] and for agar electrophoresis in citrate buffer, the method of Robinson and associates[6] was used. Quantitative estimations in zone electrophoresis were obtained by analyzing the samples in starch blocks[7] or, in a few instances, on Oxo papers[8]; in both cases a Veronal buffer, pH 8.6, μ 0.05 was used. The separated components were eluted from the starch or paper, and the hemoglobin concentrations of the eluates were determined by spectrophotometry. For free electrophoresis, about 1 per cent carboxyhemoglobin samples were analyzed in the Perkin-Elmer type of Tiselius apparatus, usually with cacodylate buffer, pH 6.5, μ 0.1. Other buffers—phosphate, pH 6.4, μ 0.04, Veronal pH 8.6, μ 0.1 or Tris, pH 7.4, μ 0.1 were used as indicated. Chromatography on columns of the ion exchange resin, Amberlite, IRC-50, was performed according to the method of Huisman and Prins.[9] Concentrations of fetal hemoglobin were determined by the method of alkali denaturation of Singer and associates[10] and solubility was measured by the method of Itano.[11]

RESULTS

Clinical Summary.—For a period of about 3 years, E. T. and her daughters have been studied at regular intervals in this hospital and have displayed

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*Another son, J. J. T., has been born recently. Hemoglobin prepared from his cord blood was analyzed by paper electrophoresis, but only types F and A were seen. However, the variant, if present, could probably not be detected in the young infant by this method.

no symptoms indicative of anemia or hemolysis. Upon physical examination, they appear normal, except that all the daughters are somewhat small, each being within the tenth to twenty-fifth percentiles for both height and weight according to her age. The fifth child is a 12-month-old boy, W. T., Jr., whose clinical picture appears ordinary. His hemoglobin pattern in paper electrophoresis, Veronal buffer, is similar to that of the mother and sisters. The father is in good health, and his hemoglobin consists entirely of type A.

The hematologic findings on the mother and children are presented in Table I.

Upon initial examination, each of the girls showed some microcytosis, but target cells were rarely seen in peripheral blood films. Microcytosis and hypochromia were particularly noticeable in the blood indices of the 2 younger girls (R. F. T. and P. A. T.). Because of the poor socioeconomic status of the family, it was thought that these abnormalities might reflect a nutritional iron deficiency. Therefore, ferrous sulfate with an ascorbic acid, Cytoferin, was prescribed in therapeutic amounts over a 6 week period, but the hemograms were not altered appreciably by this medication. However, examination approximately 1 year later showed essentially normal indices; surprisingly, at this time, one of the girls, R. F. T., had about 12 per cent target cells.

Analysis of Hemoglobin of E. T. and Her Daughters.—In paper electrophoresis, Veronal buffer, pH 8.6, or citrate buffer, pH 6.5, these hemoglobin samples were resolved into 2 components, indistinguishable from hemoglobin types A and G, respectively. A comparison of E. T.'s hemoglobin sample with those containing similar variants is shown in Fig. 1. In zone electrophoresis on agar, starch block, and Oxo paper, all in Veronal buffer, pH 8.6, the results were similar to those on filter paper. In quantitative estimations in zone electrophoresis there was 50 to 60 per cent A. The A_2 value of E. T.'s hemoglobin sample was about 3 per cent, and that of the 4 girls about 2 per cent or less.

In free electrophoresis, cacodylate buffer, pH 6.5, μ 0.1, each of these hemoglobin samples was resolved into 2 components (Fig. 2) of which the leading one, representing about two-thirds of the total area, was inseparable from hemoglobin G (or S) and the other from hemoglobin A. In a 1:1 mixture with A these proportions were reversed and in mixtures with hemoglobin S, the leading peak was increased in proportion to the amount of S added. E. T.'s hemoglobin sample was also analyzed in free electrophoresis, phosphate buffer, pH 6.4, μ 0.04, in which buffer it resolved into 2 peaks with about 75 per cent in the leading one. In this buffer, the apparent proportion of the leading component is accentuated at the expense of the slower one,[12] so that the figures for cacodylate buffer are probably a better expression of the true composition. In free electrophoresis in Tris buffer, pH 7.4, μ 0.1, E. T.'s hemoglobin sample resolved into 2 peaks of approximately equal area, inseparable from hemoglobin types A and G, respectively. In free electrophoresis, Veronal buffer pH 8.6, μ 0.1, there was one somewhat asymmetrical peak.

TABLE I. HEMATOLOGICAL DATA ON E. T. AND HER CHILDREN

PATIENT	MOTHER		CHILDREN								
	E. T.		W. T., JR.	R. F. T.		P. A. T.		J. T.		B. J. T.	
Age at the time of study (in years)	22	23*	12/12	8/12	1 9/12	2 6/12	3 7/12	3 8/12	4 8/12	4 10/12	5 10/12
Erythrocytes (million per cu. mm.)	4.7	3.7	4.7	5.0	4.4	4.9	4.3	5.2	4.8	4.6	3.83
Hemoglobin (grams per 100 ml.)	13.0	11.7	13.7	10.7	9.5	11.7	11.2	12.0	12.9	11.5	11.1
Reticulocytes (per cent)	1.6	2.5	1.5	2.0	2.0	1.2	1.5	3.0	3.0	1.2	0.6
Cell volume (per cent)	43.0	39.0	37.0	34.0	35.0	34.0	36.5	39.5	45.0	38.5	38.0
Mean corpuscular volume (cu μ)	91.7	104.0	77.5	68.0	80.0	68.9	82.0	76.0	93.5	83.7	99.5
Mean corpuscular hemoglobin ($\mu\mu$)	28.0	31.4	28.8	21.1	21.0	24.1	26.0	23.0	26.8	25.0	29.0
Mean corpuscular hemoglobin conc. (per cent)	30.2	30.0	37.0	31.4	27.2	34.2	30.8	30.4	28.8	29.8	29.2
Target cells (per cent)	0.5	0.2	0.2	0.5	11.7	0.5	3.7	0.5	0	0.5	0
Fetal hemoglobin (per cent)	0.9	—	2.8	1.1	2.0	3, 1.2†	1.6	6.2-0.5†	1.6	1.7	1.8

*Two weeks post-partum.

†Highest and lowest levels over a period of 1 year.

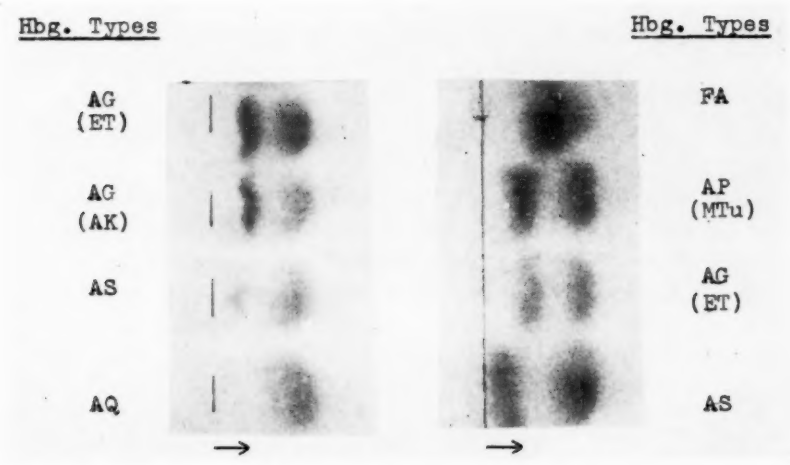

Fig. 1.—Hemoglobin variant of Patient *E. T.* compared with similar types. Paper electrophoresis patterns, Veronal buffer, pH 8.6. Initials in parentheses indicate patients' names. The sample *AQ* was kindly sent by Dr. H. Lehmann.

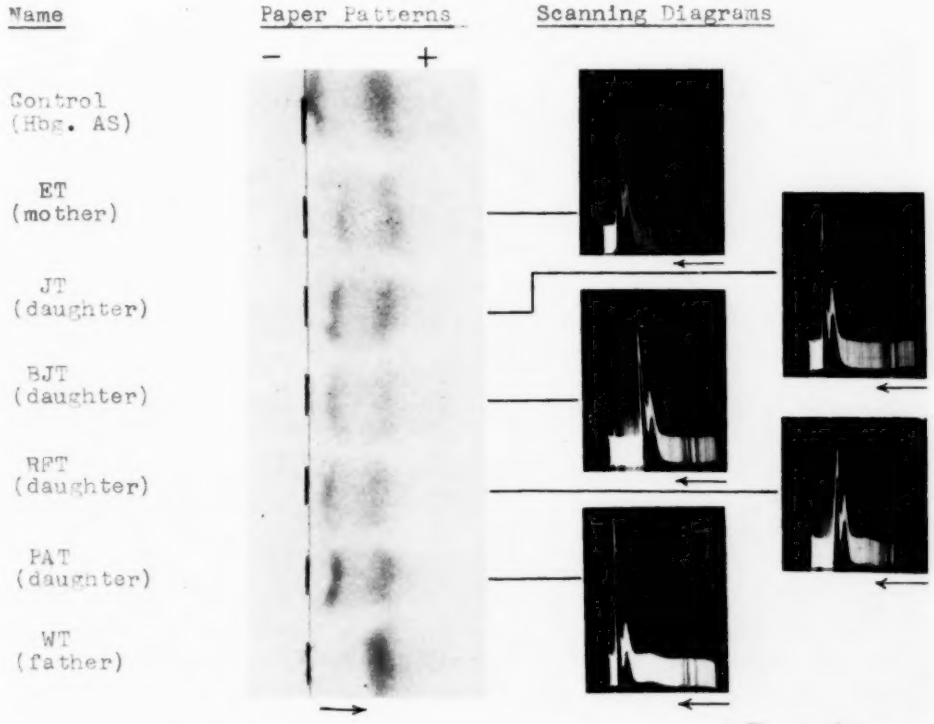

Fig. 2.—Paper electrophoresis patterns and scanning diagrams of hemoglobin samples of the T. Family. Paper electrophoresis, Veronal buffer, pH 8.6. Free electrophoresis ascending patterns, cacodylate buffer, pH 6.5, μ 0.1.

In chromatography on columns of ion exchange resin (Fig. 3), these hemoglobin samples again separated into 2 components indistinguishable from types A and G, respectively. In solubility measurements, the values fell within normal limits.

Comparison With Hemoglobin AG.—It has already been reported that in the first 2 cases of hemoglobin AG (J. R. A.[2] and A. K.[13]) the hemoglobin samples both resolved into 2 peaks of approximately equal proportions, when analyzed in free electrophoresis, cacodylate buffer, pH 6.5, μ 0.1. In each instance the leading peak was inseparable from hemoglobin S. However, in free electrophoresis, Veronal buffer, pH 8.6, μ 0.1, each sample developed into 1 peak. Recently, A. K.'s hemoglobin has been analyzed by free electrophoresis in Tris buffer, pH 7.4, μ 0.1; in this buffer there were again 2

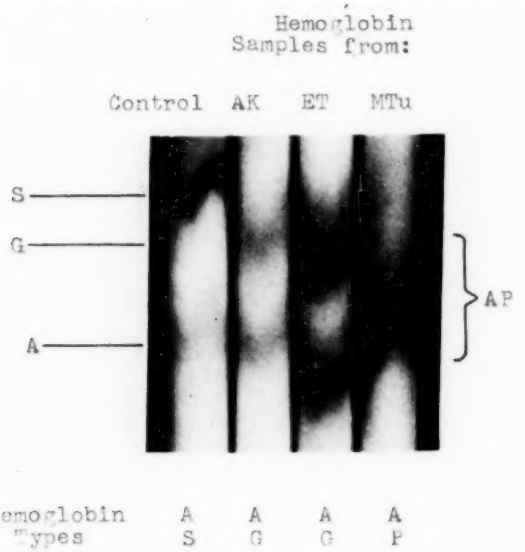

Fig. 3.—Patient *E. T.'s* hemoglobin sample compared with others in chromatography on columns of ion exchange resin.

peaks of approximately equal area. This sample was also analyzed quantitatively in zone electrophoresis, pH 8.6; here, again, there were approximately equal parts of A and G. In paper electrophoresis, citrate buffer, pH 6.5, the hemoglobin samples from A. K. and E. T. produced similar patterns. However, in agar electrophoresis, citrate buffer, there appeared to be a difference in that the variant in E. T.'s (and her daughters') samples did not separate well from hemoglobin A. Unfortunately, at this time patient A. K. became uncooperative and refused to contribute additional blood samples, so direct comparison could not be made.

DISCUSSION

The fact, that the variant in this family is the major component in free electrophoresis in cacodylate or phosphate buffers of about pH 6.5, is distinctly

unusual; almost invariably in conditions of heterozygosity for hemoglobin A and one of its variants, it is the A which comprises one half or more of the total hemoglobin (an exception has been noted in the case of hemoglobin J[14]). The one clear and consistent exception occurs in those cases in which a thalassemia gene is inherited together with the genes for hemoglobin A and a variant. The presence of thalassemia appears to suppress the formation of hemoglobin A, so that the characteristic electrophoretic pattern of the hemoglobin of patients with sickle cell–thalassemia disease or of hemoglobin C–thalassemia disease is one in which the abnormal component, S or C, is the major, and A is the minor component. In this family, the possibility of the presence of thalassemia cannot be entirely excluded, although the low values of hemoglobin F and A_2, the paucity of target cells, and the transitory nature of the mild hypochromia and microcytosis make it unlikely. Only 1 other member of E. T.'s family was available for study. This was a sister, whose hemoglobin consisted entirely of Type A.

It appears that the gene for the particular hemoglobin variant in E. T. and her children has an unusually strong quantitative expressivity, unassociated with any other distinctive abnormality. In this connection, it is interesting that in the 1 known case of homozygosity for hemoglobin G,[2] no clinical or hematologic abnormalities were found, despite the fact that the hemoglobin was entirely of type G. This variant, then, might be called a "normal" one, in contrast to those, such as S and C, whose presence in major proportions is always associated with characteristic abnormalities. Another unusual, but perhaps fortuitous, feature is that all 5 children have inherited the variant from the mother.

Finally, the discrepancy in quantitative resolution of this variant in different methods of electrophoresis is noteworthy. This discrepancy, which is probably too large to be explained by the range of error of the methods, was not found in 2 earlier cases of hemoglobin AG, and arouses suspicion regarding the structural identity of this variant with that in the earlier cases.

Recently, Benzer and associates[15] have shown that there are 3 distinct types of hemoglobin D as determined by amino acid analysis, although these are indistinguishable by all other known methods of differentiation. Other hemoglobin variants are known to be identical in one method of electrophoresis and different in another. For example, hemoglobin P[16-17] is indistinguishable from A in free electrophoresis in acid buffer (cacodylate, pH 6.2 to 6.9, μ 0.1 or phosphate pH 6.4, μ 0.04); however, in paper electrophoresis, Veronal buffer pH 8.6, P is readily separated from A but is now indistinguishable from L. Perhaps even more germane is the fact that the quantitative resolution of P (in moving boundary electrophoresis, cacodylate buffer) changes according to the nature and amount of the accompanying hemoglobin.

Eventually, all hemoglobin variants will probably be identified by analysis of the specific chemical structure.[18] Such analyses will doubtless settle the question of the precise identity of the variant in this family with hemoglobin G.

SUMMARY

A hemoglobin variant with the same electrophoretic and chromatographic mobility as hemoglobin G has been found, in conjunction with hemoglobin A, in a Negro woman and her 5 children. No distinctive clinical or hematologic abnormalities were found in these individuals. In moving boundary electrophoresis of the hemoglobin samples, the proportion of the variant is about 65 per cent in cacodylate buffer, pH 6.5, μ 0.1, about 75 per cent in phosphate buffer, pH 6.4, μ 0.04, and about 50 per cent in Tris buffer, pH 7.4, μ 0.1. In zone electrophoresis, Veronal buffer, pH 8.6, its proportion is 40 to 50 per cent. This discrepancy in quantitative resolution was not found in 2 previously reported instances of hemoglobin AG.

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STUDIES ON ERYTHROPOIESIS. XIII. A COMPARISON OF METHODS OF BIOASSAY OF ERYTHROPOIETIN IN HUMAN PLASMA

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SINCE Carnot and Deflandre's description of the erythropoietic stimulating quality of anemic serum,[1] there have been many attempts, reviewed by Grant and Root,[2] to demonstrate this property in plasma of animals and man. However, results often were equivocal or negative. A noteworthy contribution was made when incorporation of Fe^{59} by red cells, originally described by Hahn and his associates,[3] was adapted to the bioassay of erythropoietin, the erythropoietic stimulating factor in anemic plasma.[4] Modifications of this procedure have been employed to demonstrate erythropoietin in plasma of normal subjects[5] and patients with a variety of hematologic disorders.[5, 6] In previous publications,[5, 7] we have commented on the limitations of assay methods currently used and the loss of erythropoietin in fractionation of plasma prior to assay. Stohlman and Brecher[8] have also demonstrated clearly the loss of erythropoietin obtained from animal sources when the plasma was heated for 10 minutes. Ways have been sought to circumvent these losses and to increase the sensitivity of the bioassay. The purposes of this publication are (1) to report the assay of unfractionated plasma, thereby obviating erythropoietin losses by extraction, and (2) to present a comparison of the two assay animals we have employed to demonstrate erythropoietin in human plasma.

MATERIAL AND METHODS

Blood was collected from normal volunteers and anemic patients using heparin as an anticoagulant. After centrifugation in the cold, the plasma was removed and stored in the frozen state until ready for assay or fractionation. Frozen plasma was thawed at room temperature, and whole plasma or a heat-denatured extract prepared by the method of Borsook and co-workers[9] were used for assay. The plasma was acidified to pH 5.5 by the addition of 0.1 N HCl and heated in a boiling water bath for 10 minutes. The suspension was filtered through analytical filter paper (No. 595), and the precipitate resuspended in distilled water in a volume equal to the original plasma volume, heated an additional 10 minutes, filtered, again resuspended, heated, and filtered. The filtrates were combined, reduced to original volume in a rotary flash evaporator, and the pH adjusted to 7.4 by the addition of 0.1 N NaOH. This extract was then frozen until ready for assay.

Male rats of the Sprague-Dawley strain were used, 5 in each assay. The procedure of Fried and co-workers[10, 11] was followed. Young normal rats, weighing 100 to 120 grams were starved 32 hours prior to the first injection of material being assayed, and maintained in the starved state throughout the duration of the bioassay, water being

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permitted ad libitum. Six-week-old animals were hypophysectomized by the Hormone Assay Laboratories of Chicago and 1 to 2 weeks later were used for assay. Two milliliters of plasma or plasma extract was injected subcutaneously daily for 2 days. On the third day, under light ether anesthesia, a tracer dose of 1 μc of Fe^{59} citrate was given intravenously, and standards were prepared for later counting. Sixteen hours later, 1 ml. of blood was obtained by cardiac aspiration, and the Fe^{59} incorporation into red cells was calculated according to the following formula:

$$Fe^{59} \text{ uptake} = \frac{\text{net counts per ml. blood} \times 0.05 \text{ body weight}}{\text{net counts injected}}$$

In these experiments, the blood volume was assumed to be 5 per cent of the body weight.[11]

RESULTS

Normal Values.—Plasma and heat-denatured extract of plasma from 12 normal donors (all hemoglobins were between 14 and 17 gm. for men, 13 to 16 gm. for women) were assayed in both starved and hypophysectomized rats. The average results, plus or minus 1 standard deviation, are recorded

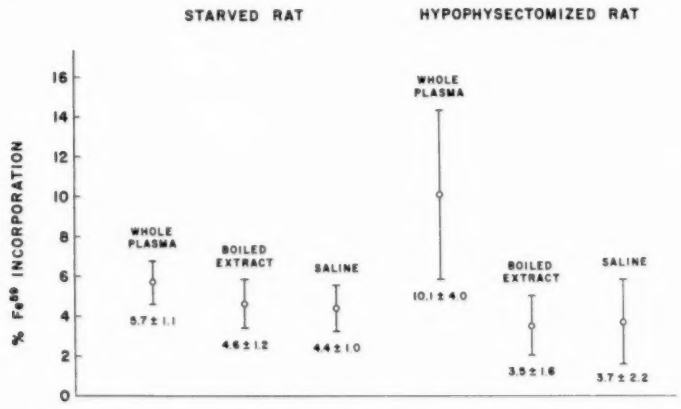

Fig. 1.—Bio-assay of erythropoietin in human plasma, control values from 12 normal donors expressed as average ±1 standard deviation.

in Fig. 1. In subsequent analyses of anemic plasmas, results exceeding these averages by more than 2 standard deviations of the latter were considered positive.

Assay of Anemic Plasmas.—Nineteen plasma specimens obtained from patients with hemoglobins under 8.5 gm. per cent, were assayed before and after protein removal by heat-denaturation in both starved and hypophysectomized rats. Diagnoses, hemoglobin levels, and the results expressed as the average of 5 animals in each assay are recorded in Table I. It will be noted that the most sensitive assay procedure involves the testing of whole plasma in starved rats, in which instance 12 of 19 specimens were positive. Only in 1 instance was a plasma negative by assay in the starved rat and positive in the hypophysectomized rat.

DISCUSSION

Since Borsook and co-workers described erythropoietic activity of the boiled extract of anemic rabbit plasma by demonstrating its stimulating effect in rats,[9] it has been customary for investigators to extract plasma in this

TABLE I. COMPARISON OF ERYTHROPOIETIN BIOASSAY PROCEDURES

DIAGNOSIS	HB.	STARVED RAT		HYPOPHYSECTOMIZED RAT	
		WHOLE PLASMA	BOILED EXTRACT	WHOLE PLASMA	BOILED EXTRACT
Normal		5.7 ± 1.1%	4.6 ± 1.2%	10.1 ± 4.0%	3.5 ± 1.6%
Myeloma	3.6	26.6 +	18.5 +	29.8 +	11.6 +
Aplastic anemia	8.1	15.5 +	9.8 +	25.1 +	12.1 +
Hodgkin's disease	3.5	27.6 +	4.8 −	27.1 +	1.9 −
Leukemia	5.0	28.8 +	4.7 −	27.3 +	3.2 −
Aplastic anemia	5.5	33.0 +	7.1 +	28.9 +	4.3 −
Myoglobinuria	7.2	12.0 +	7.8 +	21.8 +	3.4 −
Pernicious anemia	3.5	8.5 +	7.7 +	22.8 +	6.1 −
Leukemia	6.0	20.4 +	9.0 +	30.3 +	2.3 −
Acute gastrointestinal hemorrhage					
Chronic pyelonephritis	4.7	8.7 +	7.8 +	7.0 −	3.8 −
Carcinomatosis, breast	7.7	17.5 +	8.5 +	9.3 −	3.8 −
Lymphosarcoma	7.9	9.0 +	5.9 −	13.6 −	2.6 −
Leukemia	3.1	30.6 +	5.4 −	31.7 +	8.6 +
Leukemia	6.6	6.3 −	5.4 −	18.2 +	5.3 −
Lymphosarcoma	8.0	6.4 −	4.8 −	14.3 −	2.6 −
Myeloid metastasis	4.8	4.2 −	5.6 −	5.3 −	2.6 −
Myeloid metastasis	6.8	7.6 −	5.8 −	6.6 −	5.6 −
Myeloid metastasis	6.1	7.5 −	4.0 −	10.5 −	3.0 −
Leukemia	6.9	5.6 −	3.5 −	8.1 −	1.3 −
Myeloma	7.3	4.0 −	5.0 −	2.8 −	2.4 −
Summary Positive		12	8	10	3
Total		19	19	19	19

manner prior to bioassay.[5, 6, 12, 13] Indeed, even urine from anemic donors was treated in this fashion[14] prior to injection into rats in an effort to demonstrate the existence of urinary erythropoietin. We have previously reported finding erythropoietic-stimulating properties of the heat-denatured extract of human plasma obtained from many, but not all anemic donors.[5] Although no known change in the assay procedures or the hypophysectomized animals was introduced, a number of negative assays subsequently obtained were difficult to interpret if current concepts of the significance of erythropoietin are correct.[7, 15] This investigation was, therefore, designed to measure the degree to which we were losing activity when plasma was fractionated by heat-denaturation. It also appeared worth while to compare the acutely starved rat,[11] a less expensive and more resistant animal, with the hypophysectomized rat, the animal initially employed by us to demonstrate erythropoietin in human plasma.[5]

In all instances where whole plasma was found to be positive, a reduction in activity was noted after most of the protein was removed by heat-denaturation. This observation is applicable to both starved and hypophysectomized rats. In some instances, the loss of active material was so great that the heat-denatured extract was negative. We have also experienced some loss of activity when plasma proteins are removed by precipitation with ethanol in the cold or with perchloric acid. The possibility exists that the method of fractionation employing ammonium sulfate, recently reported by Lowy, Keighley, and Borsook, to yield a fraction which on a protein basis is 5 to 10 times more potent than the filtrate of the boiled anemic plasma,[16] may permit a more quantitative recovery of erythropoietin.

Rats tolerated 2 injections of the unfractionated human plasma without any demonstrable ill effects. Perhaps this is related to the difficulty with which rats form antibodies to soluble antigen.[17]

In this series of 19 plasmas tested, it would appear that the most sensitive method employs the starved rat to assay whole unfractionated plasma. This is fortunate, because this preparation is more easily maintained and less expensive than the hypophysectomized rat. It is surprising and fortunate that the nutritional value of 4 ml. of human plasma is not enough to reverse the depression of erythropoiesis in the starved rats as measured by Fe^{59} incorporation into the peripheral red cells.

Although considerable degrees of stimulation of iron incorporation into erythrocytes is obtained following injection of whole anemic plasma in the hypophysectomized rat if compared to the iron incorporation following injection of saline, these results must be evaluated in relation to a higher and more variable result obtained with normal plasma, and the difference between test values and the average normal value is therefore less striking than in the starved rat.

The stimulatory effect of whole human plasma on erythropoiesis in the hypophysectomized rat is difficult to interpret. In light of the results obtained on assay of plasma from anemic patients, it appears unlikely that the hypophysectomized rat is more sensitive than the starved rat. Therefore, it appears unlikely that the average difference between saline (3.7 per cent) and whole human plasma from normal donors (10.1 per cent) in the hypophysectomized rat is a measure of erythropoietin in normal plasma, and other explanations must be sought. Perhaps the whole human plasma supplies a sufficient quantity of one or more hormones, a deficiency of which in the hypophysectomized rat is thought to be the basis for the depression of erythropoiesis in this assay animal,[11] to account for this increased erythropoiesis. This possibility is worthy of serious consideration and warrants further investigation, because, if true, it casts serious doubt upon the validity of assaying human plasma or even plasma extracts from anemic patients in the hypophysectomized rat unless by prior fractionation substances other than erythropoietin which might be capable of stimulating erythropoiesis indirectly in the hypophysectomized rat are first removed.

The least sensitive assay procedure involved testing the heat-denatured extract of whole plasma in the hypophysectomized rat. This was a surprising finding because in an earlier study,[5] we found 10 of 18 specimens positive in the hypophysectomized rat. No change in the assay procedure was instituted, and in so far as can be determined, the animals used in this study were bred, maintained, and supplied in the same fashion as those employed previously. However, variability in assay animals has been commented on by other investigators,[18] and may also be responsible for the apparent decrease of sensitivity noted here. That the difference may at least in part be attributed to the different patients donating plasma for this study may be ascertained from the results of 2 additional assays not included in Table I because enough

plasma was not available for all 4 assays. One was an assay of plasma from a patient with hypoplastic anemic reported positive in a previous publication.[5] This plasma was stored in a deep freeze at –20° C. for 4 months. Enough was available for assay of whole plasma and the heat-denatured extract in only one animal preparation and, therefore, the hypophysectomized rat was used. The results were 51.2 ± 4.2 per cent for whole plasma and 34.6 ± 4.1 per cent for the heat-denatured extract. Twenty milliliters of plasma obtained from a child with congenital hypoplastic anemia was made available to us by Dr. Mila Pierce, Professor of the Department of Pediatrics, University of Chicago Clinics. The heat-denatured extract of this material was found to be positive, an average 13.3 ± 5.2 per cent being obtained when tested in the hypophysectomized rat (control 3.5 ± 1.6 per cent). It is interesting that a boiled extract of plasma obtained from a patient with this disease was first reported negative for erythropoietin by Piliero and associates,[14] and it was suggested that erythropoietin deficiency might be implicated in the pathogenesis of this disease. In a later publication,[18] however, these authors demonstrated erythropoietin in the patient's plasma. Perhaps loss of activity during fractionation of the initial plasma specimen contributed to the negative results.

If whole plasma is used in this bio-assay, it is important to exclude a hemolytic effect of this plasma in the assay animal, for if hemolysis were to occur, the assay results would represent a summation of the erythropoietin in the plasma being tested and the erythropoietin produced in the animal in response to its own anemia. Prior to undertaking this study, we had established to our own satisfaction the absence of a hemolytic effect of human plasma in the rat. Twenty plasma samples, stored at –16° C. were selected for this purpose because previous assays of aliquots of these specimens were positive. When assays were repeated, the hematocrits of the assay animals were also determined. In no instance was there a fall in the average hematocrit of the assay animals when compared to the hematocrit of control assay animals receiving either saline or plasma from normal donors. Hence, employing the technique of assay as described here, we were of the opinion that a hemolytic effect of 2 daily injections of plasma obtained from anemic patients was excluded.

However, since completion of this study, Lowy and co-workers[19] have described the hemolytic effect in starved rats of 3 daily injections of plasma obtained from rabbits made anemic by phenylhydrazine. It would therefore appear desirable to incorporate routine hemoglobin or hematocrit determinations of assay animals into the procedure particularly when whole plasma of a species different from that of the assay animal is being employed.

The significance of negative assays, discussed in detail in a previous publication,[5] continues to remain obscure. The role of erythropoietin deficiency in the pathogenesis of anemia cannot be adequately assessed until more quantitative and sensitive assay methods are perfected or erythropoietin is available for clinical testing.

SUMMARY

1. The plasma and heat-denatured extract of plasma of 19 anemic patients were assayed for erythropoietic-stimulating properties in hypophysectomized and starved rats of the Sprague-Dawley strain. The results were compared to normal values established by testing normal plasma and a heat-denatured extract of it, obtained from 12 donors.

2. The most sensitive procedure in this study was the assay of whole plasma in the starved rat, where 12 of the 19 specimens were positive.

3. Appreciable losses of activity result when plasma proteins are removed by heat-denaturation.

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REACTIONS IN THE LATEX-FIXATION TEST FOR RHEUMATOID ARTHRITIS WITH SERUM OF SYPHILITIC INDIVIDUALS

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WITH THE TECHNICAL ASSISTANCE OF MARGIT RUBIN

REACTIONS have been reported in latex-fixation tests for rheumatoid arthritis of serum from individuals known to have syphilis and of serum yielding reactions in serologic tests for syphilis. Rheins and co-workers[1] obtained reactions in 7 out of 24 cases of syphilis; Rothermich and Philips[2] reported reactions in 2 cases of seropositive, treated, latent syphilis, but no reactions in a treated case of neurosyphilis nor in 4 seropositive cases with syphilitic status undetermined. More recently, Peltier and Christian,[3] while employing the tannated-erythrocyte hemagglutination test for rheumatoid arthritis, obtained 16 reactions with 147 syphilitic sera. Latex-fixation test results were also given in 14 out of the 16 cases. Eleven reactions were obtained with the latter test. Data given indicate that the factor responsible for reactions is not syphilitic reagin.

The present paper reports the results of further investigation of the frequency and intensity of the reactions obtained in the latex-fixation test with syphilitic sera. A series of 232 specimens from individuals with syphilis was studied. Data are also given on an additional case of serologic reactions suggestive of rheumatoid arthritis in a syphilitic individual, which was studied earlier. This case, which in part stimulated this study, was followed for approximately 5 years, with interesting results.

MATERIALS AND METHODS

Syphilitic sera were selected from those submitted for control of treatment; all gave 4+ reactions in the New York State slide flocculation test for syphilis and titers of 0 to 140 in the New York State complement-fixation test for syphilis. Each of these specimens was tested by the Hyland-RA test for rheumatoid arthritis. This commercially distributed slide-test modification of the latex-fixation procedure is very rapidly performed. The manufacturer's instructions were followed.

Reacting sera were further examined by a modification of the original tube technique of Singer and Plotz[4] in accordance with mimeographed instructions distributed by the authors. The modifications consisted primarily of the substitution of the glycine-buffered saline solution, pH 8.2, as diluent, for borate-buffered saline solution, pH 8.2, and the definition of a "positive" test as one giving agglutination in serum dilutions greater than 1:20, i.e., beyond tube 1, after 90 minutes' incubation at 56° C., or greater than 1:160, i.e., beyond tube 4, after overnight refrigeration. The degree of agglutination was recorded on a scale indicated in Table I. The last 2+ or greater reading was considered the end point.

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TABLE I. SYPHILITIC SERA (15 OF 232) FOUND TO REACT IN THE LATEX-FIXATION TEST FOR RHEUMATOID ARTHRITIS

PATIENT	TITER COMPLEMENT-FIXATION TEST FOR SYPHILIS	HYLAND-RA SLIDE TEST	SINGER AND PLOTZ TUBE LATEX-FIXATION TEST: INCUBATED 90 MINUTES AT 56° C. AGGLUTINATION* IN TUBE NO.†									PLUS OVERNIGHT AT 3° TO 6° C. AGGLUTINATION IN TUBE NO.									HISTORY OR EVIDENCE OF RHEUMATOID ARTHRITIS OR OTHER COLLAGEN DISEASE
			1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	
T. H.	2	Reactive	3+	2+	2+	2+	+	±	–	–	–	4+	4+	3+	2+	+	±	±	–	–	None
Lv.	2	Reactive	2+	2+	2+	2+	+	±	–	–	–	2+	2+	2+	2+	+	±	±	–	–	None
M. C.	0	Reactive	4+	3+	3+	2+	+	+	±	–	–	4+	4+	3+	3+	2+	2+	+	±	–	None
Le.	Anticomple-mentary	Reactive	2+	2+	2+	+	±	±	–	–	–	4+	3+	3+	2+	+	±	±	–	–	None
R.	13	Reactive	3+	3+	2+	2+	+	±	–	–	–	4+	4+	3+	2+	+	+	±	–	–	None
M. B.	4	Reactive	3+	3+	3+	2+	+	+	±	–	–	4+	4+	4+	3+	2+	+	+	±	–	Very mild form of asymptomatic hypertrophic arthritis of both hands
P. P.	2	Reactive	2+	+	+	±	±	±	–	–	–	3+	2+	2+	+	+	+	±	±	–	None
M. Br.	0	Reactive	4+	4+	3+	2+	2+	+	+	±	–	4+	4+	4+	3+	2+	+	+	±	±	None
C. B.	6	Reactive	2+	2+	+	+	±	–	–	–	–	3+	2+	+	+	±	–	–	–	–	None
G. W.	9	Reactive	3+	3+	3+	2+	2+	+	+	±	±	4+	4+	4+	3+	2+	+	+	+	±	None
D. C.	58	Weak reaction	±	±	±	–	–	–	–	–	–	2+	2+	+	+	+	±	–	–	–	None
M.	0	Weak reaction	+	±	±	±	±	–	–	–	–	3+	2+	2+	+	+	±	±	–	–	None
T.	12	Weak reaction	+	±	±	–	–	–	–	–	–	2+	+	+	±	±	±	–	–	–	None
B.	8	Weak reaction	+	+	±	±	–	–	–	–	–	2+	+	+	±	±	–	–	–	–	None
J. C.	8	Weak reaction	2+	+	+	+	±	–	–	–	–	3+	2+	+	+	+	±	±	–	–	None

*4+, Particles agglutinated in flakes, background clear; 3+, particles agglutinated in flakes, background slightly turbid; 2+, particles agglutinated in small flakes, background moderately turbid; +, definite but granular agglutination; ±, questionable agglutination; –, particles in turbid, homogeneous suspension without visible aggregates.

†Dilutions are serial twofold beginning with initial 1:20 (before addition of latex particles) through 1:5,120.

In the examination of 155 specimens from 148 individuals submitted to this laboratory for serologic tests for rheumatoid arthritis, the slide and tube procedures appeared to be similar in sensitivity.[5] In the Hyland-RA slide test, 39 specimens gave a reaction, 8 gave a weak reaction, 104 gave no reaction, and 4 showed variable results. In the Singer and Plotz tube test, 45 gave a reaction, 108 gave no reaction, and 2 showed variable results.

In order to determine, for purposes of comparison, the extent of reactivity of presumably normal sera, 200 premarital specimens yielding no reaction in the New York State slide flocculation test were examined by both procedures. In the case of this group only the initial (1:20) dilution of the sera was tested in the Singer and Plotz tube test.

Gamma globulin (dried Squibb plasma fraction II) was obtained through the courtesy of the American Red Cross Blood Program; Dow polystyrene latex particles were received from the Arthritis and Rheumatism Foundation.

RESULTS

None of the 200 presumably normal specimens reacted in the Hyland-RA test. In the Singer and Plotz tube test, the maximum agglutination observed in this group was ± after 90 minutes' incubation at 56° C. and + after refrigeration overnight.

Of the 232 specimens from individuals with syphilis, 10 gave reactions and 5 gave weak reactions in the Hyland-RA slide test. Results with these specimens are shown in Table I. Nine of the 10 specimens which elicited reactions in this test also yielded reactions in the tube latex test according to the above criteria, but none of the sera that were weakly reactive in the former test were reactive in the latter. It should be noted, however, that agglutination in the tube test with all 15 specimens was greater than that encountered with any of the 200 presumably normal specimens examined.

Inquiries from physicians who submitted these specimens revealed that only 1 of the patients whose serum reacted in the latex test had symptoms in any way suggestive of rheumatoid arthritis; this was a 72-year-old woman with a very mild form of asymptomatic hypertrophic arthritis of both hands.

DISCUSSION

The results strongly suggest that false-positive latex-fixation tests for rheumatoid arthritis occur occasionally with serum of patients with syphilis. The behavior of the serum in 1 case of syphilis suggests, however, the possibility that reactions in tests for rheumatoid arthritis may anticipate clinically apparent disease and may not necessarily indicate a false-positive reaction due to syphilis.

Case History.—M. M., a 46-year-old Negro woman, was hospitalized in 1947 with a diagnosis of psychosis with syphilis of the central nervous system, meningo-encephalitic type. Specimens of blood examined that year showed titers as high as 200 in the complement-fixation test for syphilis. The New York State macroprecipitation and microprecipitation tests for syphilis and the V.D.R.L. slide test were also reactive. With cerebrospinal fluid a titer of greater than 10 was obtained in the complement-fixation test for syphilis and a first zone or "paretic" type of curve in the colloidal gold test. The patient was treated with malaria and arsenical drugs; also twice with penicillin. By 1959, the complement-fixation titer of the blood had fallen to 5 and that of the cerebrospinal fluid to 3. The first serologic abnormality suggestive of rheumatoid arthritis was noted in September, 1953, when a specimen of blood agglutinated sheep red blood cells in the complement-fixation test for

syphilis but failed to agglutinate unsensitized cells in the conventional test for heterophile antibodies. Similar results were obtained with blood specimens collected in December, 1953, and again in December, 1954. Euglobulin fractions from each of the latter 2 specimens, precipitated in the cold by the method of Svartz and Schlossmann,[6] reacted in a hemagglutination test for rheumatoid arthritis. In reply to an inquiry made in December, 1954, information was received that the patient did not have a history or evidence of rheumatoid arthritis.

A latex-fixation test was performed on a specimen of blood collected in July, 1958, and a titer of 1:1,280 was obtained. Inquiry was again made regarding the patient's status and the following clinical evidence of rheumatoid arthritis was reported:

"Proximal finger joints are especially affected and at the present they are showing varying degrees of ankylosis. X-rays showed narrowing of the joints in her hands (carpal bone joints and interphalangeal joints). This patient's shoulder joints showed narrowing with some calcification. Her right knee shows narrowing with eburnation of the articular margins. X-ray suggests evidence of rheumatoid arthritis with possibly superimposed osteoarthritis of the right knee."

Further questioning revealed that clinical evidence of rheumatoid arthritis first appeared in late 1954, at which time the patient complained of pains in the knees.

Although psychotic patients often minimize symptoms, it appears that the serologic findings may have anticipated clinical symptoms by about a year. In view of this possibility, it seems necessary to follow the 15 cases previously discussed and any other cases of syphilis with serologic evidence of rheumatoid arthritis for several years before reactions in latex tests can be said with certainty to be falsely positive.

SUMMARY

Fifteen of 232 specimens from individuals with syphilis reacted in latex-fixation tests for rheumatoid arthritis to an extent not ordinarily encountered with normal serum. Fourteen of these individuals had no history or evidence of rheumatoid arthritis; the fifteenth had a very mild form of asymptomatic, hypertrophic arthritis of both hands.

While these findings strongly suggest that syphilis may cause false-positive reactions in latex-fixation tests for rheumatoid arthritis, the circumstances surrounding an additional case indicate that syphilitic patients showing serologic evidence of rheumatoid arthritis will have to be followed for several years to rule out impending clinical arthritic disease.

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ESTIMATION OF LEFT-TO-RIGHT SHUNT FROM ARTERIAL DILUTION CURVES

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ARTERIAL dilution curves recorded for patients with left-to-right shunts, following injection of the indicator into the venous circulation, are characteristically distorted. The distortion consists of a decrease in peak concentration, prolongation of the disappearance phase frequently associated with a break on the disappearance slope, and decrease or absence of the systemic recirculation peak.[1, 2] Since the distortion is caused by the recirculation of the dyed blood through the lungs, the degree of distortion is greater in the presence of larger shunts.[2]

In 1954, Broadbent and Wood[2] demonstrated a positive correlation between the magnitude of the left-to-right shunt and the ratio of the disappearance time to the build-up time (DT/BT) of arterial dilution curves recorded after injection of an indicator into an antecubital vein. The disappearance time was obtained by extrapolation directly on the photographic recording of the disappearance phase of the curve along the visually best-fitting straight line to its intersection with the base line of zero concentration of the indicator.[1] This method, although reasonably satisfactory for dilution curves recorded after injections into a peripheral vein, usually cannot be applied to curves recorded after injections of the indicator into more central sites, such as the pulmonary arteries. This is due to the fact that such curves are more sharply defined than those obtained after injection of the indicator into a peripheral vein[3] and thus, when a left-to-right shunt is present, show a definite break in the disappearance phase of the curve with a change in the slope which makes extrapolation of the disappearance phase of the dilution curve by a single visually best-fitting straight line impossible.

The present report describes a simple method of estimating the magnitude of left-to-right shunts from arterial dilution curves based on ratios of the concentrations of indicator measured at specific instants on the dilution curve. This method can be applied successfully to curves recorded after injections of indicator at selected peripheral or central sites, and the measurements required are not subject to errors of interpretation.

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MATERIALS AND METHODS

Arterial dilution curves, obtained after injection of the indicator into the central venous circulation of a normal subject and a patient with a left-to-right shunt, are compared in Fig. 1. Except for the short interval after the peak concentration has been reached, the concentration values during the disappearance phase are larger in the patient with a shunt, and it might be expected that the larger the shunt the greater will be the concentration at a given instant after the peak. However, the absolute concentrations also depend on other factors, such as the amount of indicator injected, size of the patient, cardiac output, and the volume of blood between the site of injection and the sampling site. These factors which vary from patient to patient and influence the contour and amplitude of the curve[2, 4] have to be compensated for, as suggested by Broadbent and Wood,[2] if the degree of distortion of indicator-dilution curves related to the magnitude of the left-to-right shunt in different patients is to be validly compared. In the present study, compensation for variations in timing and amplitude of the curve caused by the nonspecific factors mentioned was obtained by measuring concentrations on the disappearance phase of the curve at a time equal

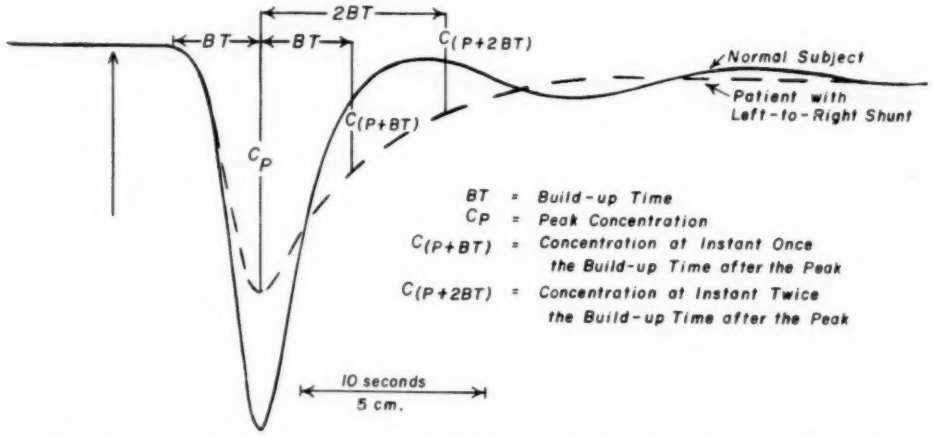

Fig. 1.—Comparison of arterial dilution curves obtained following injection of the indicator into the venous circulation of a normal subject and a patient with a left-to-right shunt. The concentration values at instants of time once [$C_{(P+BT)}$] and twice [$C_{(P+2BT)}$] the build-up time (BT) after the peak concentration (C_P) are measured as illustrated. Note that these concentrations are greater in the patient with a left-to-right shunt than in the normal subject.

to the build-up time after the peak deflection [$C_{(P+BT)}$] and at a time equal to twice the build-up time after the peak deflection [$C_{(P+2BT)}$] as illustrated in Fig. 1, and then by dividing the concentrations at these specific points on the disappearance phase of the curve by the peak concentration (C_P) of the curve. The values obtained, designated as disappearance ratios, were then correlated with the magnitude of the shunt. The disappearance ratio [$C_{(P+BT)}/C_P$] has been used previously by Fox and Wood[5] to quantitate the prolongation of the disappearance slope caused by a left-to-right shunt in comparing dilution curves obtained using Evans blue and methylene blue as indicators and by Woodward and co-workers[6] and Marshall and co-workers[7] to differentiate patients with predominant mitral stenosis and regurgitation.

In addition, the ratio of the least concentration to the recirculation concentration (C_L/C_R) was measured in all the curves recorded after injection into the pulmonary artery, in which there was a discernible systemic recirculation peak. In curves in which no recirculation peak was apparent the C_L/C_R ratio was considered to be greater than 1.0. This concentration ratio has been used previously to distinguish between patients with predominant mitral stenosis and regurgitation.[8]

One hundred dye-dilution curves of 47 patients (22 males and 25 females) with left-to-right shunts were studied. The mean age of the group was 28 years with a range of 3 to 56 years. Fifteen patients were less than 20 years of age, and 3 were less than 10 years old. None of the patients included in this study showed evidence of a right-to-left shunt either from data on oxygen saturation of arterial blood or from the dilution curves. The group included 28 patients with interatrial communications, 15 with interventricular communications, and 4 with patent ductus arteriosus. In addition, 55 curves from 31 normal subjects were analyzed to obtain the range of values for these ratios encountered in healthy individuals. Also twenty-one dilution curves from 14 patients with stenotic valvular disease were analyzed to obtain data concerning the effect of varying degrees of congestive heart disease on these ratios. The averages and ranges of the systemic blood flow were 3.5 (2.3 to 6.5), 3.4 (1.7 to 8.0), and 2.3 (1.5 to 3.5) L. per minute per square meter, respectively, for normal subjects, patients with left-to-right shunts, and patients with stenotic valvular disease.

Right-heart catheterization was performed in all patients and subjects with the techniques previously described which included measurements of intracardiac and vascular pressures and the oxygen saturation of multiple blood samples withdrawn from these sites.[9, 10] The indicator was injected into the main pulmonary artery, one of its branches, the right ventricle, or a vena cava. Because of the phenomenon of preferential shunting from the right as compared to the left lung in patients with interatrial communications,[11] only dilution curves obtained after injections into the right ventricle or the venae cavae were used in those patients. Evans blue dye (T-1824),* in doses of 7.5 to 15.0 mg., was used as the indicator for 53 injections into 26 patients with left-to-right shunts, for 55 injections into 31 normal subjects, and for 21 injections into 14 patients with stenotic valvular disease. Cardio-green dye (indocyanine green),† in doses of 2.5 to 7.5 mg., was used for 47 injections into 21 patients with left-to-right shunts. When cardio-green was given, the patients were breathing air. When Evans blue was given, all the normal individuals, all the patients with stenotic valvular disease, and 17 patients with left-to-right shunts were breathing 100 per cent oxygen, whereas 4 patients with shunts were breathing air. The injections of Evans blue were made into the remaining 5 patients with left-to-right shunts both while they were breathing air and while they were breathing 100 per cent oxygen.

The curves were recorded by means of a cuvette oximeter attached to an indwelling 20-gauge needle placed in the radial artery. The concentrations were measured from the photographic records in relative units of deflection in centimeters and were converted into absolute concentration values expressed in milligrams per liter from the calibration curves of the oximeter. The disappearance ratios, $C_{(P+RT)}/C_P$ and $C_{(P+2RT)}/C_P$, were calculated both from relative concentrations (deflections in centimeters) and from the absolute concentrations.

The shunt, expressed as per cent of pulmonary flow, was calculated from the formula:

$$\text{Shunt} = \frac{(Q_P - Q_S) \times 100}{Q_P}$$

where Q_P equals pulmonary flow and Q_S equals systemic flow, calculated by the Fick method. The Fick determination was made while the patient was breathing air or oxygen, whichever was used during the recording of the dilution curves for that patient or subject. Since none of the patients had evidence of a right-to-left shunt, the oxygen content of blood in the pulmonary vein was assumed to equal that in the radial artery. In 13 patients with atrial septal defect in whom the oxygen saturation of inferior caval blood was not measured, saturation of mixed venous blood was assumed to equal that in the superior vena cava plus 3 per cent.[13] There was no apparent difference in the correlation of concentration ratios with the shunt values between patients in whom only superior caval saturation was measured and those in whom both vena caval saturations were known.

*The Evans blue dye was supplied by the Warner-Chilcott Corporation.

†This dye which has also been designated tricarbocyanine dye II[12] was supplied by the Eastman Kodak Laboratories and Hynson, Westcott & Dunning, Inc., of Baltimore, Md.

In 3 patients with interatrial communications and 3 patients with interventricular communications, the per cent of shunt was calculated from the indicator-dilution curves recorded simultaneously from the right side of the heart and from a systemic artery as described by Wood and co-workers.[11]

RESULTS

The disappearance ratios derived from the relative concentrations (deflections in centimeters) were plotted against the ratios of absolute concentrations calculated from the same dye curves (Fig. 2). The deviation from the line of identity was small, and there was no apparent difference between the scatter for the $C_{(P + BT)}/C_P$ and $C_{(P + 2BT)}/C_P$ ratios. Consequently, in the subsequent data only the values calculated from deflections in centimeters are presented. When ratios derived from concentrations expressed in absolute units (milligrams per liter) were used, no significant differences in the correlations were evident.

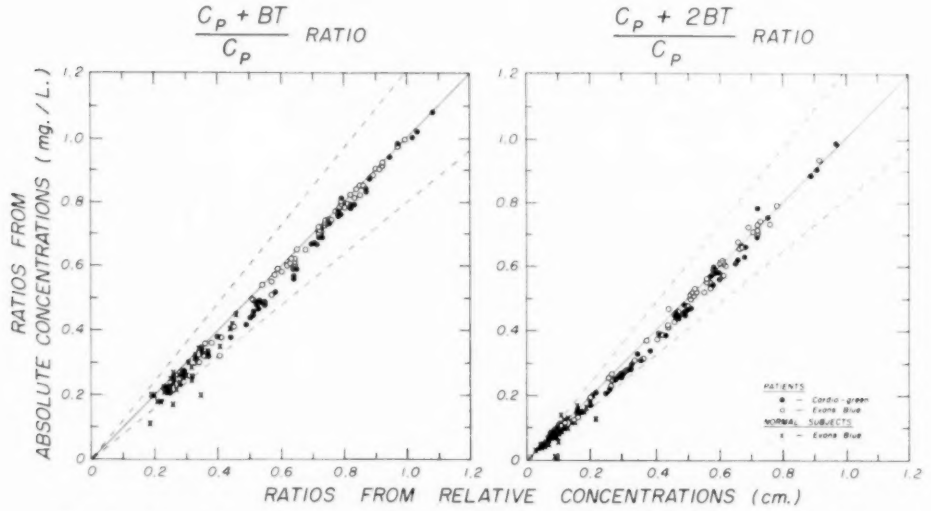

Fig. 2.—Comparison of the disappearance ratios calculated from relative deflection in centimeters and absolute (mg. per liter) concentrations. The interrupted lines encompass a variability of ± 20 per cent from the line of identity (solid line). Note the slight systematic difference due to the nonlinearity of the calibration curve of the oximeters in terms of dye concentration.

This is of practical importance, since the time required to obtain the ratio of deflections measured directly from the recorded curve is much less than that required to convert these deflections to concentrations and to calculate the ratios.

The possible effect upon the disappearance ratios of variations in the injection site on the right side of the heart was investigated by plotting the ratios measured from the curves recorded after injection of the indicator into the superior vena cava against the values obtained in the same patients following injections into other sites (Fig. 3, upper panels). Variation in the injection site on the right side of the heart caused no apparent systematic difference, for either ratio, in the values obtained from the patients with left-to-right shunts. Although there was no systematic difference for the $C_{(P + BT)}/C_P$ ratio of normal subjects, the ratio $C_{(P + 2BT)}/C_P$ of normal subjects and both ratios of

patients with valvular stenotic heart disease were larger after injection into the superior vena cava than after injection into the main pulmonary artery.

This systematic difference in these patients, most of whom had mitral stenosis, may be related to the larger than usual volume of blood in the right ventricle and atrium coupled with the low cardiac output in such patients and in addition to the probable presence of subclinical degrees of tricuspid regurgitation in some of the patients associated with pulmonary hypertension, congestive

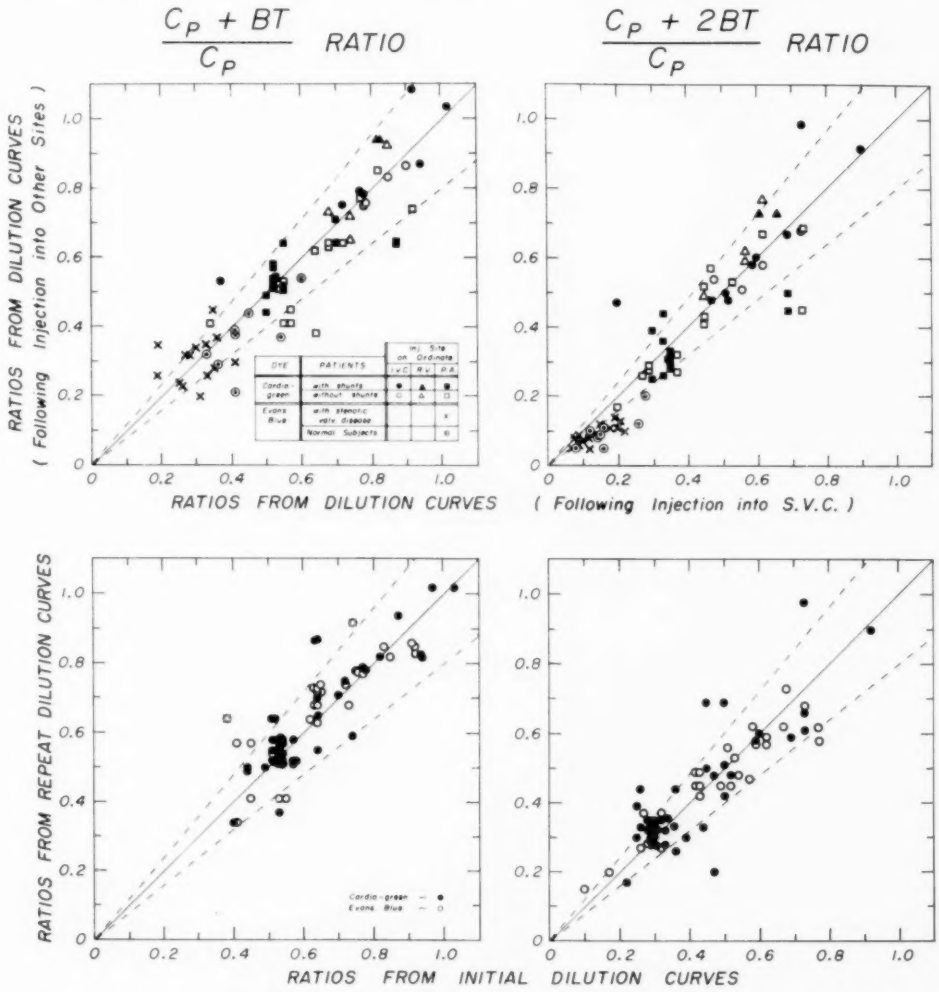

Fig. 3.—Comparison of disappearance ratios obtained following injection of the indicator into different sites on the right side of the circulation (upper panel) and variability between disappearance ratios determined from repeat dilution curves at different times during the cardiac catheterization procedures (lower panel). The interrupted lines encompass a variability of ± 20 per cent from the line of identity (solid line). Note: (1) that in the patients with left-to-right shunts there was no systematic difference between the ratios obtained following injection of the indicator into the superior vena cava and into other more central sites on the right side of the heart; however, the values of ratios $C_{(P + 2BT)}/C_P$ and $C_{(P + BT)}/C_P$ calculated from the curves recorded following the injection into the superior vena cava of the patients with stenotic valvular heart disease and the values of ratios $C_{(P + 2BT)}/C_P$ in normal subjects were greater than those obtained after injection into the main pulmonary artery; (2) that there was no systemic difference between the ratios obtained from the initial and from the repeat curves. The average time interval between these pairs of curves was 28 minutes with a standard deviation of 20 minutes.

heart failure, and cardiomegaly. The cardiac index was less than 2.0 L./min./M.^{2} in all but 2 of these patients who had unusually high disappearance ratios. This difference in ratios associated with injections into the pulmonary artery and vena cava was small and occurred only in the lower range of values. It is unlikely that this variation with injection sites in the central circulation would affect appreciably the correlation between the ratios and the magnitude of the shunt, since the ratios in patients with left-to-right shunts in the great majority of cases lie above the range in which this difference is apparent.

In order to gain information concerning the reproducibility of disappearance ratios measured from repeated dilution curves in the same patient and to determine whether these ratios would vary with time during the catheterization procedure, the ratios obtained from multiple injections performed in the same patients were analyzed. For each patient the ratio obtained from each initial curve was plotted against the ratio of all subsequently recorded curves as illustrated in Fig. 3 (lower panels). The average interval between the 70 pairs of curves was 28 minutes with a standard deviation of 20 minutes. There was no systematic change in these ratios with time. The ratios for normal subjects and patients with stenotic valvular heart disease are not shown because all injections into the main pulmonary artery preceded injections into the superior vena cava and the results appear in the upper panels of Fig. 3.

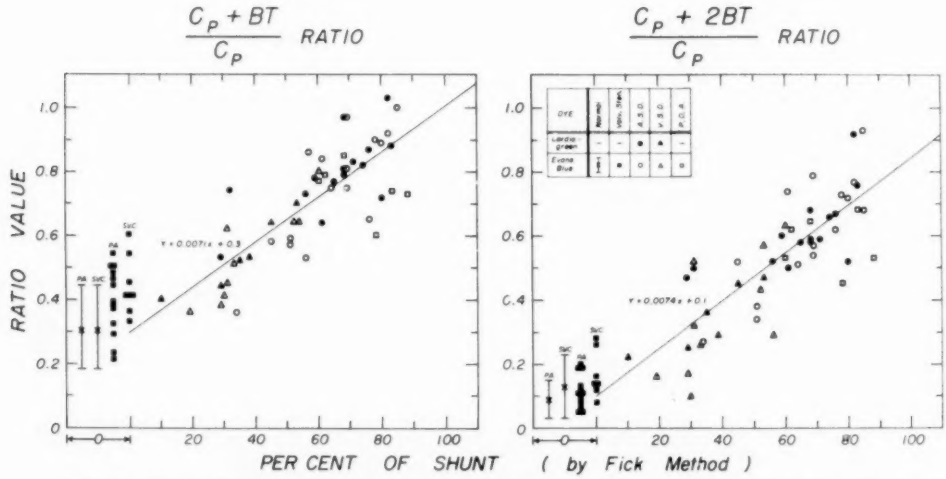

Fig. 4.—Correlation of the magnitude of the left-to-right shunt calculated from the data on oxygen saturation by the Fick method with the disappearance ratios of the arterial dilution curves. The regression lines are calculated from the ratios of normal subjects and patients with left-to-right shunts. The vertical bars represent the 95 per cent band range for normal subjects. Note that no difference is apparent when cardio-green and Evans blue dyes were used as the indicators, nor for the cases with the various types of cardiac defects.

The disappearance ratios obtained from the dilution curves nearest in time to the determination of flow by the Fick method were plotted against the magnitude of the shunt calculated from the saturation data (Fig. 4). The average interval between these dye curves and the Fick determinations was 30 minutes with a standard deviation of 25 minutes. The 5 patients in whom both determinations were performed while they were breathing air and also while they

were breathing 100 per cent oxygen are represented by 2 points on the graph. A significant correlation of the ratios with the calculated magnitude of the shunt is evident. There is no apparent difference in the scatter of points around the calculated regression lines for patients with various types of defects. Furthermore, the scatter is similar in patients in whom cardio-green and Evans blue dye were used. There is no striking difference in the correlation of the magnitude of the shunt with the 2 disappearance ratios. There was no systematic difference in the correlations obtained between patients more than 20 years of age and those less than 20 years of age.

This correlation between the magnitude of the disappearance ratio and of the left-to-right shunt as determined from data on oxygen content can be used for the dual purpose of detecting the presence of a left-to-right shunt from an arterial dilution curve and for estimating the magnitude of such a shunt when it is known to be present.

Estimation of the magnitude of the shunt can be made directly from the regression lines shown in Fig. 4 by reading off the per cent of shunt on the abscissa corresponding to the measured disappearance ratios or by calculating the per cent of left-to-right shunt from the regression equations:

$$\text{Per cent L-R} = 141 \left[\frac{C_{(P + BT)}}{C_P}\right] - 42$$

$$\text{Per cent L-R} = 135 \left[\frac{C_{(P + 2BT)}}{C_P}\right] - 14$$

in which per cent L-R represents the left-to-right shunt expressed as per cent of pulmonary flow. The other symbols have been defined previously. The standard deviations of the differences between the values of the shunts determined by the Fick and by this dye method were 8.9 and 9.6 per cent for $C_{(P + BT)}/C_P$ and $C_{(P + 2BT)}/C_P$ ratios, respectively. This variability was not significantly different for the dilution curves in which cardio-green and Evans blue dye were used. The standard deviation of the differences between the values for the shunt determined from the same dilution curve by the 2 disappearance ratios was 4.9 per cent.

An estimate of the sensitivity of detection of small left-to-right shunts from the disappearance ratio is provided by the degree of overlapping between ratios obtained from normal subjects and the patients with small left-to-right shunts. Of the 47 patients studied, 4 had the ratios $C_{(P + 2BT)}/C_P$ that were within the 95 per cent band range of values for normal subjects. The magnitude of the left-to-right shunts in these patients was 30 per cent of the pulmonary blood flow or less. The ratios $C_{(P + BT)}/C_P$ for 6 patients with left-to-right shunts of 35 per cent or less fell in the normal range (Fig. 4). These data indicate, therefore, that shunts of less than 35 per cent of the pulmonary flow cannot be consistently detected by an abnormal increase in the disappearance ratio.

As illustrated in Fig. 4, there was greater overlapping between the disappearance ratios of patients with left-to-right shunts and those with stenotic valvular heart disease than between patients with left-to-right shunts and the normal subjects. However, the overlapping in the case of $C_{(P + 2BT)}/C_P$ ratio

from curves recorded after injection into pulmonary arteries was small and comparable to the overlapping between normal subjects and patients with left-to-right shunts.

A more sensitive method for detection of small left-to-right shunts from arterial dilution curves is provided by a ratio of least concentration to systemic recirculation (C_L/C_R) of the dilution curves recorded after injection into the pulmonary artery. The C_L/C_R ratio was clearly abnormal for the dilution curves recorded from each of the 35 patients with left-to-right shunts of greater than 35 per cent of pulmonary flow. In fact, this ratio was greater than 1.0 in 31 of these patients since a discernible systemic recirculation peak was evident on the dilution curves recorded from only 4 of these 35 patients, whereas an evident recirculation peak was present on the dilution curves recorded from all of the 12 patients with shunts of 35 per cent or less. The C_L/C_R ratios calculated from the curves of these 12 patients ranged from 0.53 to 1.0 and were uniformly increased above the average values obtained in normal subjects or cardiac patients with stenotic lesions but without valvular regurgitation; only one value (0.53) was less than 0.65, which is the upper limit of range found in individuals without shunts or regurgitation (Table I). This degree of sensitivity in detecting small left-to-right shunts does not compare unfavorably with that provided by conventional cardiac catheterization based on determinations of differences in blood oxygen saturations from different sites in the right heart, since, under favorable circumstances, the minimal left-to-right shunt detectable by the latter method ranges from approximately 10 to 20 per cent of pulmonary blood flow.[13]

TABLE I. AVERAGE AND VARIABILITY OF LEAST CONCENTRATION TO THE SYSTEMIC RECIRCULATION RATIOS (C_L/C_R) OBTAINED FROM ARTERIAL DILUTION CURVES (INJECTION SITE: PULMONARY ARTERY; SAMPLING SITE: RADIAL ARTERY)

	HEALTHY SUBJECTS	CARDIAC PATIENTS* WITHOUT LEFT-TO-RIGHT SHUNTS	PATIENTS WITH LEFT-TO-RIGHT SHUNTS OF:	
			35 PER CENT OR LESS	MORE THAN 35 PER CENT
Number	23	14	12	35
Average ratio	0.42	0.34	0.84	> 1.0
Range	0.26-0.60	0.16-0.65	0.53-1.0	0.90 to > 1.0
Standard deviation	0.09	0.19	0.14	—

*Patients suspected of having valvular regurgitation on the basis of clinical or surgical findings were not included.

DISCUSSION

The objective of this study was to develop, if possible, a simple method of detecting and estimating the magnitude of a left-to-right shunt from dilution curves recorded from a systemic artery following a sudden single injection of indicator into the central circulation. The lack of an accurate standard of reference was one of the chief difficulties in this study, since the error in the accepted method of detecting and measuring left-to-right shunts by differences in oxygen content of the blood at various sites in the central circulation is, in itself, in the range of 10 to 20 per cent. The finding that the standard deviation of the differences between values for left-to-right shunts determined by differences

in oxygen content and by this dye method was less than 10 per cent suggests that the error in the dye method probably does not exceed that of the oxygen content method. It is possible that by averaging the values of the shunt obtained from the 2 disappearance ratios, the error of this dye method may be reduced even further.

During the period of this investigation, an independent and sensitive method for detection and quantitation of the left-to-right shunt became available. This method is based on dilution curves recorded from the right side of the heart at a site downstream from the shunt after injection of the indicator into the distal part of the pulmonary circulation.[12] The magnitude of a left-to-right shunt can be calculated from a "venous" dilution curve and the curve recorded simultaneously from a systemic artery as described by Wood and co-workers.[14] Comparison of the values of the left-to-right shunt determined by this method from venous dye curves recorded in 6 patients with values determined by the disappearance ratio method from the arterial dilution curves recorded from these same patients is shown in Fig. 5. The relatively small degree of variability obtained between the values of the shunt determined by these 2 independent indicator-dilution methods probably provides a better indication of the reliability of the disappearance ratio method than does comparison with values for the shunt determined from differences in oxygen content.

Although the disappearance ratios of normal subjects and patients with left-to-right shunts of 35 per cent or less overlap, when a clearly defined break on the disappearance slope is present the magnitude of the shunt may be estimated by the disappearance ratio method even if the actual ratios should fall within the range of normal. Furthermore, calculation of the ratio of the least

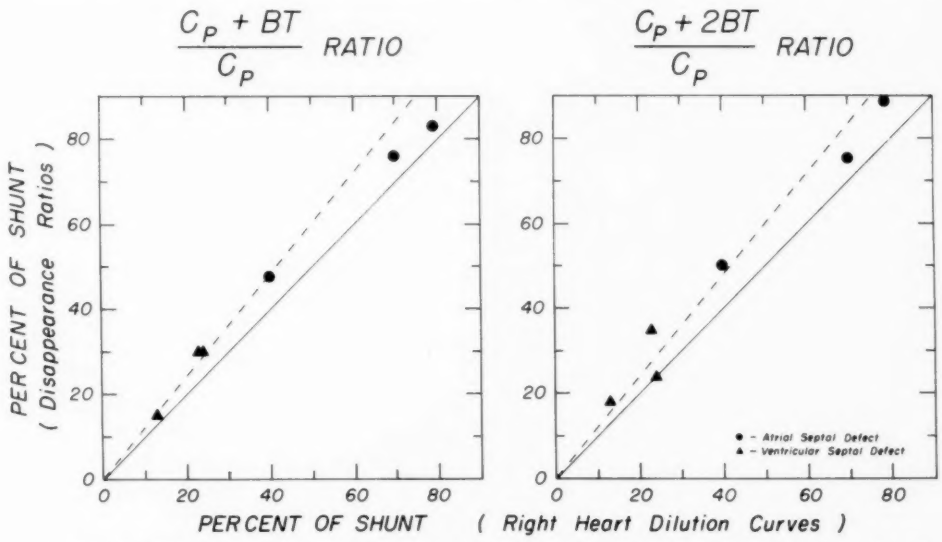

Fig. 5.—Correlation between the values of the left-to-right shunt obtained from the disappearance ratios of arterial dilution curves and the magnitude of the shunt calculated from dilution curves recorded simultaneously from the right side of the heart and from a systemic artery. The interrupted line encompasses a variability of ± 20 per cent from the line of identity (solid line).

concentration to the systemic recirculation concentration (C_L/C_R) is of value in those borderline cases in which visual inspection of the dilution curve does not indicate clearly whether the disappearance phase is distorted significantly. The minimal left-to-right shunt, detectable by an abnormal increase in the C_L/C_R ratio of the arterial dilution curve recorded after injection into the pulmonary artery, apparently is of the same order of magnitude as the 10 to 20 per cent left-to-right shunt which can be demonstrated under favorable circumstances by detecting the increase in oxygen content produced in blood on the right side of the heart.[13]

The dye method has the advantage in that the dilution curve is recorded over a period of less than 1 minute and the measurements and calculations required to estimate the left-to-right shunts can be completed in a matter of minutes. Pulmonary blood flow also can be estimated from this dilution curve by the method of Dow[15] or that of Ramirez de Arellano and associates.[16] No manometric analyses of gases in the blood or measurements of respiratory exchange of gases are necessary for these estimations.

If the injection of indicator is made into the superior or inferior vena cava, the presence of a right-to-left shunt will be revealed and, if it is present, an estimate of its magnitude can be made.[17] Such a dilution curve provides an excellent basis for judging the relative volumes of systemic and pulmonary blood flows and hence for selection of such patients for cardiac surgery if the anatomic nature of the defect is known.[18]

A single dilution curve of this type is, however, of little value in localizing the site of a cardiac defect; it provides no information concerning intracardiac pressures, and, furthermore, the distortion produced by a left-to-right shunt and valvular regurgitation may be difficult or impossible to distinguish. The presence of appreciable bidirectional shunts or valvular regurgitation precludes quantitation of the shunts and blood flow by this dye method.

The degree of sensitivity in detecting left-to-right shunts provided by this method, even if combined with calculation of the C_L/C_R ratio, leaves much to be desired and has been improved on by the recently reported foreign gas techniques[19-21] and particularly by the method of recording dilution curves from the right heart downstream from the shunt following injection of indicator into the distal pulmonary circulation.[12, 14] Although the technique of recording dilution curves from the right side of the heart is considerably more cumbersome than the dilution technique described herein, it is the procedure of choice if precise localization and quantitation of a small left-to-right shunt are required. Diagnosis and elucidation of the hemodynamics associated with multiple or complicated defects require combinations of various techniques and methods available in a fully equipped cardiac catheterization laboratory and are beyond the scope of the relatively simple dilution methods described herein.

SUMMARY

The disappearance ratios of concentration of indicator [$C_{(P+BT)}/C_P$ and $C_{(P+2BT)}/C_P$] were calculated from more than 100 arterial dilution curves recorded after injection of the indicator into the right heart or pulmonary artery

by measuring the deflection occurring on the disappearance phase of dilution curves at a time equal to the build-up time after the peak deflection [$C_{(P + BT)}$] and at a time equal to twice the build-up time after the peak concentration [$C_{(P + 2BT)}$] and by dividing by the peak deflection (C_P). One hundred of these curves were recorded from 47 patients with left-to-right shunts due to various types of cardiac defects. In addition, 21 dilution curves were recorded from 14 cardiac patients with stenotic valvular disease not associated with left-to-right shunts or valvular regurgitation, and 55 dilution curves were recorded from 31 healthy human subjects. The ratio of the least concentration to the systemic recirculation concentration (C_L/C_R) also was determined from each of the curves that were recorded after injection into the pulmonary artery.

A positive correlation was demonstrated between the magnitude of the 2 disappearance ratios and the magnitude of the left-to-right shunt determined by differences in oxygen content of blood samples from the right side of the heart obtained at right-heart catheterization. This correlation can be used in patients with unidirectional left-to-right shunts and without valvular regurgitation as a basis for estimating the relative magnitude of the shunt. The standard deviations of the differences between the values of the shunt estimated by the $C_{(P + BT)}/C_P$ and $C_{(P + 2BT)}/C_P$ ratios and by the conventional method based on the differences in oxygen content of blood from the right heart and great veins were 9 and 10 per cent, respectively. These ratios can be calculated in a matter of minutes from deflections measured directly from the recorded dilution curve without conversion to absolute units of concentrations. The values are reproducible and, in patients with left-to-right shunts, do not change significantly with variations in the site of injection on the right side of the heart. It is concluded that this dilution method is considerably simpler than the oxygen content method for estimating the magnitude of left-to-right shunts and that its accuracy is satisfactory for most clinical purposes.

A discernible systemic recirculation peak was absent from all but 4 of the arterial dilution curves recorded after injection into pulmonary arteries of 35 patients with left-to-right shunts of greater than 35 per cent of pulmonary flow, and was present on the curves from all 12 patients with shunts of 35 per cent or less. The C_L/C_R ratios for all but 1 of these 12 patients were larger than the highest values obtained in healthy subjects or patients with stenotic valvular disease. It is concluded that in the absence of valvular regurgitation the presence of an abnormally high C_L/C_R ratio in the curves recorded after injection into pulmonary arteries is a reasonably sensitive measure for detecting a small left-to-right shunt.

This study was made possible by the unstinting cooperation of many technical and professional colleagues. The authors are especially indebted to Drs. H. J. C. Swan, I. J. Fox, and A. David, and Mrs. J. Frank, Miss L. Cronin, and Mr. W. F. Sutterer for their help and interest in this study.

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A FATAL HUMAN CASE OF RABIES FOLLOWING THE BITE OF A RABID BAT (LASIONYCTERIS NOCTIVAGANS)

Isolation and Identification of the Virus From Vector and Victim

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IT IS now generally recognized that nonsanguivorous bats are susceptible to infection with rabies virus and that some may harbor the virus as symptomless carriers, since their saliva is infectious.[1] Although it has been reported[2] that experimentally infected bats (the cave bat, *Myotis lucifugus*) can transmit the virus to laboratory animals by bite, there is no absolutely unequivocal evidence that infected insectivorous or fructivorous bats can transmit the virus to man by biting.* So far as we are aware, the literature records 3 instances of human rabies associated with exposure to bats. The first case, reported by Sulkin and Greve,[3] involved a woman who developed symptoms suggestive of bulbar poliomyelitis 3 weeks after being bitten by a bat which she went to examine because it was lying on the ground and appeared dead. The patient died within a few days after onset of illness and at autopsy Negri bodies were seen in the brain tissue. The bat, which was not identified, was not examined for infection with rabies virus. The second case occurred in India.[4] The patient was bitten by a bat which he attempted to rescue from being stoned by a group of boys, and developed rabies 3 months later; the bat apparently was not identified, nor was it examined for the presence of rabies virus. The third fatality occurred in an individual who handled bats in the laboratory and in caves harboring bat populations; there was no history of a bite by a bat or any other animal.[5] The portal of entry of the virus in this individual was thought to be an area of chronic eruption on the skin.

The purpose of this communication is to present laboratory evidence relating a fatal human case of rabies to the bite of an infected bat. The clinical and epidemiologic features of this case will be described in a separate report by Humphrey and associates.[6] Briefly, for present purposes, the patient, a 53-year-old woman, was bitten (on the finger) by a silver-haired bat (*Lasionycteris noctivagans*, an insectivorous species†) which she picked up from the ground where it was lying, ostensibly injured. Microscopic examination

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*After this paper was submitted for publication, there appeared the report of Bell,[25] describing the transmission of rabies to suckling mice by the bite of a naturally infected insectivorous bat, *Myotis californicus californicus*.

†We are indebted to Mr. Keith F. Murray, Mammalogist, Bureau of Vector Control, California State Department of Public Health, for the identification of this specimen.

of the bat brain revealed the presence of Negri bodies. The victim received rabies hyperimmune serum and a full course of rabies vaccine* (started on the fourth day after the exposure) but succumbed to rabies some 2 months after the bites were inflicted. Rabies virus, which had been recovered earlier from the brain of the bat, was also recovered from the human victim.

MATERIALS AND METHODS

Methods for the Detection of Virus.—Methods employed in this laboratory for the diagnosis of rabies have been described previously.[8]

Smear or touch preparations were prepared from brain material in the usual fashion, stained by Seller's method,[9] and examined for the presence of Negri bodies.

For virus isolation purposes, a tissue fragment was ground in a mortar and made into a 10 per cent suspension in 0.85 per cent salt solution. After horizontal centrifugation at 2,500 r.p.m. for 10 minutes, the supernatant fluid was removed, and penicillin and streptomycin were added in appropriate amounts to give final concentrations of 1,000 and 5,000 units, respectively, per milliliter. The suspension was held at room temperature for 30 minutes and then inoculated intracerebrally in 0.03 ml. amounts into a group of 8 albino Swiss mice, 4 weeks of age. Aliquots of this final suspension were tested for bacteriologic sterility.

Identification of Viral Isolate.—As a verification that the agent recovered was rabies virus, the isolate was examined in the neutralization test. Infected mouse brains were made into a 20 per cent suspension in a diluent consisting of 0.75 per cent bovine albumin in buffered saline solution, centrifuged horizontally at 2,500 r.p.m. for 20 minutes and the supernatant fluid used to prepare serial tenfold dilutions in the same diluent. To 0.2 ml. of virus dilution was added 0.2 ml. of undiluted rabies hyperimmune serum (equine). The resultant mixture was well shaken and inoculated intracerebrally in 0.03 ml. amounts into mice, 4 weeks of age; each mixture was inoculated into a group of 6 mice. A similar series of virus-serum mixtures, using normal horse serum, was prepared and inoculated into mice. Identification of the isolate as rabies virus was based on a neutralization index of 2 or more, i.e., the neutralization of at least 100 mouse LD_{50} of the unknown virus.

Tissues for Histologic Examination.—Portions of cerebrum, cerebellum, midbrain, and brain stem from the patient were received fixed in Zenker's solution. A small block from each specimen was thoroughly washed, dehydrated and cleared, and imbedded in paraffin. Sections 5μ thick were cut on a rotary microtome and stained with hematoxylin and eosin, with Giemsa's stain, and by Schleifstein's method.[10]

RESULTS

Intensive examination of smears prepared from the human brain, and stained by Seller's method, failed to reveal the presence of typical Negri bodies; while inclusion bodies were present, they did not contain the characteristic basophilic granules. Tissue sections from the region of the thalamus and the hippocampus, and stained by the Giemsa or Schleifstein methods, showed the presence of intracytoplasmic inclusions in the neurons but no structures with the characteristic morphologic and tinctorial appearance of Negri bodies.

The histopathologic picture was that of a diffuse nonsuppurative encephalomyelitis. The midbrain and brain stem were especially affected. There was a well-marked infiltration of cells into the perivascular spaces, the infiltrates consisting chiefly of lymphocytes with some polymorphonuclear and

*Duck-embryo type.[7]

microglial cells. Perivascular cuffing in the medulla and the thalamic region was pronounced, and there were many foci of necrobiosis, with collections of microglial cells, lymphocytes, and polymorphonuclear cells. Neuronal degeneration and neuronophagia were seen in the midbrain and brain stem.

Blocks of unfixed tissue representing the frontal lobe, the cerebellum, the hippocampus, and the cervical cord were available for virus isolation purposes; each specimen was sent to the laboratory in a separate container. A 10 per cent suspension of each specimen was made, and inoculated intracerebrally into mice. Material from all 4 areas of the brain proved infective; mice inoculated with the cervical cord became ill after an incubation period of 8 days, and those inoculated with the cerebellum, the hippocampus, and the cerebrum became ill after incubation periods of 10, 12, and 14 days, respectively. The brains of ill mice from each of the 4 inoculation groups were found to contain Negri bodies. Re-isolation of the agent was undertaken in mice, using the original cervical cord specimen, and a Negrigenic agent was obtained after an incubation period of 13 days.

That the agent recovered from the human brain tissue was rabies virus was further established by means of a neutralization test employing rabies hyperimmune serum. The intracerebral LD_{50} of the virus in the presence of normal horse serum was $1 \times 10^{-4.4}$, and in the presence of rabies antiserum it was $1 \times 10^{-1.0}$; the neutralization index therefore was 3.4, i.e., this specific immune serum neutralized approximately 2,500 LD_{50} of the viral isolate.

DISCUSSION

The earliest association of bats with disease apparently dates from 1908, when ranchers in southern Brazil noted that vampire bats were attacking livestock in the daytime, and that the bitten animals subsequently succumbed to a paralytic disease.[11] In 1911, Carini[12] identified this paralytic disease as rabies and suggested that it might be transmitted to livestock by vampire bats. Some years later (1921) Haupt and Rehaag[13] also concluded that the repeated epizootics of paralytic disease among livestock in southern Brazil were outbreaks of rabies transmitted by sanguivorous bats. In 1931, Hurst and Pawan[14] reported an outbreak of human rabies with the simultaneous occurrence of rabies in cattle; in man, as in cattle, the disease took the paralytic form. The authors suspected that the virus was transmitted to both man and cattle by the vampire bat (*Desmodus rotundus murinus* Wagner); subsequently, this bat was definitely incriminated as a vector of human rabies.[15]

Rabies in the Order Chiroptera is not limited to vampire bats, however. Thus, Haupt and Rehaag[13] reported the occurrence of natural infection in a fruit-eating bat from Brazil, and Pawan[15, 16] reported natural infection in both fructivorous and insectivorous species in Trinidad.

Generally speaking, the bats of the New World can be divided into three large categories according to their feeding habits, viz., sanguivorous, fructivorous, or insectivorous. The insectivorous bats may be subdivided into free-living and colonial species, the former living an individual existence in trees and bushes, the latter living as members of a colony within caves, belfries,

atties, etc. Apparently only insectivorous bats are found in the United States, the sanguivorous and fructivorous species being confined to Latin America.[17]

In the United States, the first isolation of rabies virus from an insectivorous bat was made by Venters and associates[18] in Florida in 1953. Since that time, many additional isolations of virus have been made from insectivorous bats in Florida[19] and other states.[17, 20-23] However, reported attacks on human beings by nonsanguivorous bats have been few; specifically, there have been only 6 such reported instances of which we are aware. Two were reported by Venters and associates,[18] one by Witte,[20] one by Grimes and co-workers,[24] one by Sulkin and Greve,[3] and one from India.[4] In 3 instances,[18, 20, and 24] the biting bats were shown to be rabid; the victims, who received antirabies vaccine, remained well.* In the fourth instance, mentioned by Venters and associates,[18] the individual was bitten when examining bats, and 6 days later developed severe headache, nausea and fever; the symptoms subsided after 4 days.* The fifth case is that reported by Sulkin and Greve[3] of a woman who was bitten by a bat and 3 weeks later developed what was considered to be a bulbar poliomyelitis. The patient succumbed to the illness, and histopathologic examination of the brain revealed the presence of Negri bodies. The offending bat was not available for identification or for viral studies. However, "the history together with the histopathologic findings strongly suggests that the bat bite was the source of the rabies infection."[3] The sixth case, reported from India,[4] is that of a man who developed rabies 3 months after being bitten by a bat—the bat was not identified or examined for infection with rabies virus.

While the incident described by Sulkin and Greve strongly points to a cause and effect relationship between the bat bite and fatal rabies in the human victim, the episode described in this paper not only supports their presumption, but serves definitely to incriminate a free-living insectivorous bat (the silver-haired bat, *Lasionycteris noctivagans*) as a vector of the virus to man and possibly to other animals as well. The bat involved was definitely infected with rabies virus; Negri bodies were seen in smears of the brain stained by Seller's method and the virus was isolated from the brain. The virus was likewise recovered from the brain of the patient who, despite the administration of rabies hyperimmune serum and a complete prophylactic course of rabies vaccine, succumbed to infection.

SUMMARY

A fatal case of human rabies following the bite of a silver-haired bat, *Lasionycteris noctivagans*, a free-living insectivorous species, is reported. This is believed to be the first instance in which evidence for a cause and effect relationship of bite to infection is complete, since the virus was recovered from both the vector and the victim.

*The offending bat was not examined for rabies virus, and the clinical course was such as to suggest that some agent other than rabies virus was etiologically responsible for the illness. Also, it is uncertain whether this illness was a consequence of the bat bite, even though there was no other apparent source.[18] It is of interest that the patient on whom the case report by Venters and co-workers[18] is based, and who was bitten by a bat proved to be rabid, suffered a similar illness which developed suddenly 28 hours after the bite; the symptoms subsided within hours and rabies immunization was started.

The patient succumbed to infection despite prophylactic treatment with rabies hyperimmune serum and a complete course of rabies vaccine, duck-embryo type.

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THE INFLUENCE OF STORAGE ON ERYTHROCYTE SURVIVAL IN BLOOD OBTAINED FROM DONORS WITH SICKLE CELL TRAIT

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REPORTS of splenic infarction during air flight,[1-6] pulmonary infarction,[7] spontaneous hematuria,[8] and impairment of renal function in individuals with sickle cell trait[9] indicate that this heterozygous hemoglobinopathy is not always an innocuous, asymptomatic, genetic abnormality.[10] Nevertheless, since unrestricted activity and normal life expectancy are commonplace among individuals with sickle cell trait, the incidence of overt disease must be very small. Because a large segment of the population[10-11] possesses AS hemoglobinopathy, it seemed appropriate to study the influence of ordinary blood bank storage of sickle cell trait blood upon erythrocyte survival.

METHODS

One donor with ordinary (AA) hemoglobin and 2 donors with AS hemoglobinopathy, who were normal in all other respects, were selected. Five hundred milliliters of venous blood from each donor was aspirated into an evacuated blood bottle previously prepared with 120 ml. ACD anticoagulant. The usual blood grouping and typing as well as serologic tests for syphilis were performed. The blood was stored in the blood bank refrigerator at 4° C.

On the first, eighth, fifteenth, and twenty-second days of storage, 30 ml. aliquots of blood were removed from a bottle which contained erythrocytes with AA hemoglobin, and on the first, eighth, fifteenth, and twenty-first days, in 1 instance, and on the first, eighth, seventeenth, and twentieth days, in the other instance, 30 ml. of blood was aspirated from each of 2 bottles containing erythrocytes with AS hemoglobin; aseptic technique was used. Each aliquot was labeled with Cr^{51} as sodium chromate, according to a modification of the method of Read and co-workers.[12] Immediately afterward, the labeled erythrocytes, suspended in their native plasma, were injected intravenously into a group- and type-specific volunteer, whose hemoglobin genotype was AA. Survival of the homologous hemoglobin AA and hemoglobin AS erythrocytes, respectively, was determined.

RESULTS

Fig. 1 is a composite graph demonstrating erythrocyte survival of blood, from a donor with hemoglobin genotype AA, in each of 4 compatible recipients whose hemoglobin genotypes were AA. Transfusion was after 1, 8, 15, and 22 days of storage. Blood bank storage exerted no prejudicial effect upon erythrocyte survival. There was no significant difference in the half-life ($T\frac{1}{2}$) values; this indicates similar survival following these periods of storage.

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Fig. 2 presents similar data which were obtained following the transfusion of stored blood from Donor 1, whose hemoglobin genotype is AS. After 1, 8, and 15 days of storage had elapsed, erythrocyte survival in compatible recipients with hemoglobin genotype AS was normal and similar to that of stored hemoglobin AA blood. After 21 days of storage, erythrocyte survival as expressed by the half-life appears to be within the normal range, although survival is

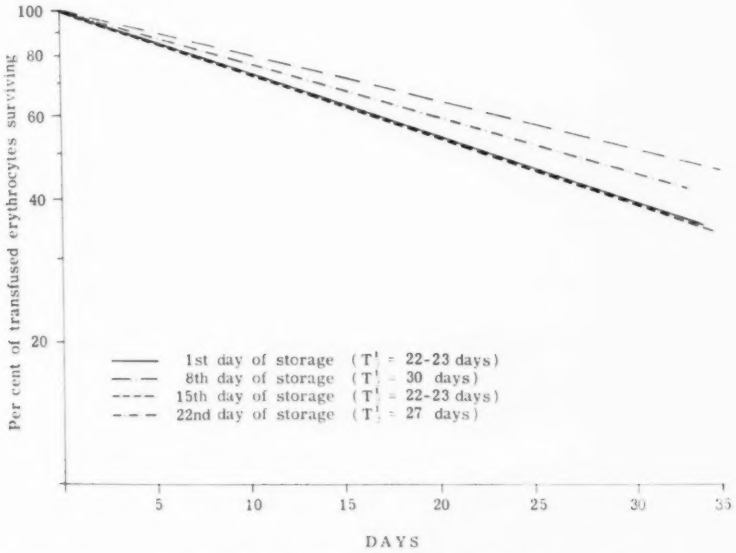

Fig. 1.—Posttransfusion survival of homologous erythrocytes with AA hemoglobin following blood bank storage of 1, 8, 15, and 22 days.

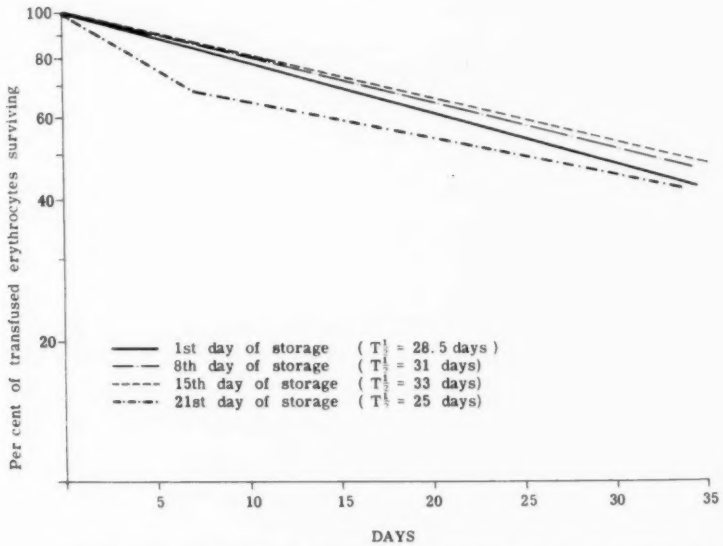

Fig. 2.—Posttransfusion survival of homologous erythrocytes from Donor 1 (AS hemoglobinopathy) following blood bank storage of 1, 8, 15, and 21 days.

slightly shortened when compared to blood stored for 1, 8, or 15 days. Also, there is a suggestion that 2 erythrocyte populations are present: a small component of effete cells whose half-life is about 13 days, and a larger component of normally resistant cells whose half-life is about 27 days.

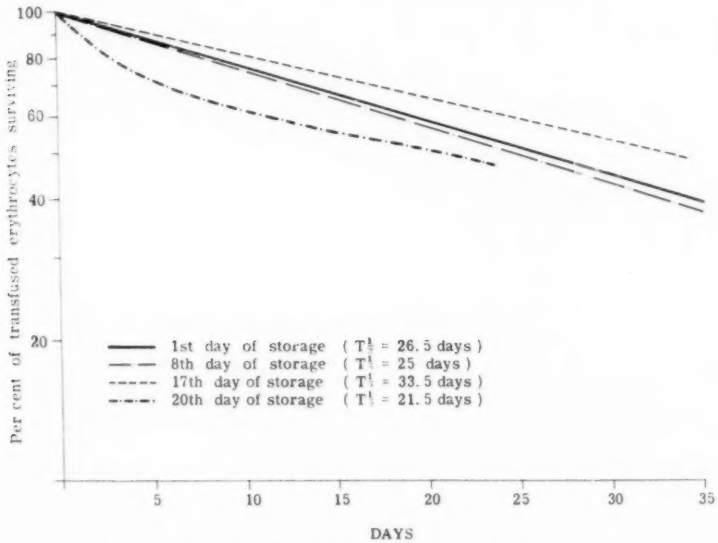

Fig. 3.—Posttransfusion survival of homologous erythrocytes from Donor 2 (AS hemoglobinopathy) following blood bank storage of 1, 8, 17, and 20 days.

Similar results are presented in Fig. 3, in which survival of erythrocytes from Donor 2 with AS hemoglobinopathy is plotted. Following 1, 8, and 17 days of storage, erythrocyte viability appears to be normal and unchanging. Again, however, following 20 days of storage, there is a suggestion of a slight shortening of erythrocyte life, which may be associated with 2 populations of cells. There is a smaller population of cells with a half-life of about 13 days, and a larger population of resistant cells with a half-life of about 24 days.

DISCUSSION

It has been previously reported that erythrocytes from individuals with sickle cell trait possess normal survival characteristics.[13, 14] However, these studies were performed with the use of freshly shed blood. The possibility that blood bank storage might produce significant shortening of erythrocyte survival remained; therefore, the present study was undertaken.

The survival time of stored AS erythrocytes appeared to be completely normal and almost identical following 1, 8, 15, and 17 days of storage, but following 20 to 21 days of storage, there was a suggestion of initial shortening of survival. A two-component survival pattern was apparently responsible for the initial sagging of the curve, and this suggests that after such prolonged storage, there may be at least 2 cell populations; a relatively smaller population of exceedingly fragile cells and a much larger population of normally viable cells.

Comparison of these curves with those obtained from a similar experiment using stored blood from a donor with hemoglobin genotype AA reveals no essential difference, except after 20 to 21 days of storage. On the basis of these observations, it seems quite certain that blood bank storage imposes no significantly greater prejudicial effect upon erythrocytes from AS donors than upon those from donors with genetically "normal" hemoglobin. The corollary of this observation is that sickle cell trait blood may be stored in blood banks for the accepted periods of time and that it may be transfused without fear of provoking a hemolytic reaction.

SUMMARY

Blood collected from donors with sickle cell trait was stored under routine blood bank conditions. Erythrocyte survival studies, begun 1, 8, 15, and 17 days following collection, revealed no significant reduction over what might be expected in stored blood from donors with the hemoglobin genotype AA.

Following 20 to 21 days of storage of AS erythrocytes, there may be 2 populations of cells present; a smaller relatively fragile population and a considerably larger normally resistant population.

Blood obtained from sickle cell trait donors provides no additional risk of hemolysis to the recipient and may, therefore, be treated in a routine fashion in the blood bank.

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MAGNESIUM BALANCE STUDIES IN CHRONIC ALCOHOLISM*

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THE magnesium deficiency syndrome in man[1-11] is characterized by fine and coarse tremor of the extremities, choreiform and athetoid movements of all muscles, fasciculations of the tongue, convulsions, sweating, and varying degrees of mental clouding, progressing to delirium and coma.

The serum magnesium concentration is usually low in patients with this syndrome but may be normal. Serum potassium concentration is a poor index of body stores of potassium under certain circumstances.[12] It is possible, therefore, that a similar discrepancy between serum concentration and body stores might occur in the instance of the other major intracellular cation.

Low serum magnesium concentration is found most often in patients with chronic alcoholism,[2, 4, 6, 9] in patients who have been maintained for long periods on magnesium free parenteral fluids after operation or while seriously ill with vomiting or diarrhea,[1, 2, 4, 5, 11] in patients with acute pancreatitis,[7] idiopathic steatorrhea,[11] malnutrition from various causes,[13] hyperparathyroidism[10, 14] osteolytic bone disease,[15] diabetic acidosis following treatment,[16] and hyperthyroidism.

A normal person eating a general diet does not develop magnesium depletion since magnesium is present in most foods. Meat, dairy products, cereal grains, and nuts have a high magnesium content.[18]

Magnesium balance studies were done during the recovery of a group of tremulous and hyperkinetic individuals with chronic alcoholism to determine whether total body magnesium deficiency existed in such patients.

METHODS

Seven adult men, hospitalized for treatment of peptic ulcer, served as controls. Magnesium balances were measured over periods ranging from 4 to 8 days. The diet consisted of homogenized milk or half cream and half milk. Toast and egg supplements were given to some patients as indicated in tables. Two of the 7 control subjects were given intramuscular magnesium sulfate on 2 and 4 day periods, respectively, during the study. The magnesium intakes in Tables II, III, and V are recorded as "measured" intake for amounts actually measured by chemical analysis and as "calculated" intake for the amounts calculated from food tables.[18] All intakes in Table IV were measured intakes. Patients were given no antispasmodic drugs. The constipating effects of the diet and inactivity were controlled with methyl cellulose granules in water daily.

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The magnesium balance of 11 tremulous patients with chronic alcoholism was measured over 6 to 10 day periods. In 7, the diet was identical to the diet given the control patients. The 4 other patients (Table IV, C. F., R. E., W. L., and R. H.) were given a more palatable liquid diet composed of whole milk, orange juice, beef-vegetable puree, and chocolate malted milk. The magnesium content of the diet was measured daily. Five of the alcoholic patients were given magnesium sulfate supplements intramuscularly.

All urine was collected daily. Stools were passed into waxed cardboard containers. Urine, serum, and liquid food aliquots were analyzed for magnesium by the titan yellow method of Kolthoff as modified by Garner.[19] Stools were pooled and mixed in a Waring Blendor. Aliquots were processed by a wet ash method using concentrated nitric acid for digestion.[20] The magnesium of the stool filtrate was measured by the titan yellow method.

RESULTS

Values for magnesium content of stool specimens obtained by the Shohl modification of Stolte's dry ash method[20] were compared with values obtained by the wet ash method (Table I). The results by the 2 methods agree within the limits of error of the colorimetric method. The wet ash method was used for the balance studies. The mean serum magnesium concentration of 36 normal subjects was 1.81±0.13 mEq. per liter.

In the 5 control patients with magnesium intake as diet alone (Table II), the net magnesium balance over periods ranging from 4 to 8 days varied from a negative balance of .27 mEq. to a positive value of 17 mEq. In 2 control patients, who received magnesium sulfate injections in addition to diet, the added magnesium was excreted in the urine. The net magnesium balances were plus 5 and plus 10 mEq. (Table III).

TABLE I. COMPARISON OF WET ASH AND DRY ASH METHODS OF STOOL PROCESSING

PATIENT	DAYS POOLED	STOOL MAGNESIUM (mEq.)	
		DRY ASH	WET ASH
S. K.	3	36.0	36.0
R. H.	4	52.0	54.0
G. P.	3	33.0	35.2

TABLE II. FIVE CONTROL SUBJECTS TREATED WITH DIET ALONE

PATIENT	DURATION OF STUDY	ORAL INTAKE (mEq.)		OUTPUT (mEq.)		BALANCE (mEq.)
		MEASURED	CALCULATED	URINE	FECES	
M. S.	5	102.0	5.4	30.4	59.8	+17.3
G. D.	4	97.7		24.9	82.0	−9.2
S. K.	5	87.9	17.6	17.5	85.0	−3.1
G. T.	6	110.2		50.2	87.0	−27.0
G. S.	8	154.4		104.9	69.0	−19.5
Mean		113.9 (Intake)		122.1 (Excretion)		

TABLE III. CONTROL SUBJECTS TREATED WITH DIET AND $MgSO_4$

PATIENT	DURATION OF STUDY	INTAKE (mEq.)			OUTPUT (mEq.)		BALANCE (mEq.)
		ORAL		I. M. $MgSO_4$	URINE	FECES	
		MEASURED	CALCULATED				
F. C.	6	75.6	30.6	48.0	108.3	41.1	+4.8
G. P.	6	65.4	62.8	96.0	134.6	79.6	+10.0
Mean		189.2 (Intake)			181.8 (Excretion)		

TABLE IV. ALCOHOLIC PATIENTS TREATED WITH DIET ALONE

PATIENT	WEIGHT (KG.)	DURATION OF STUDY (DAYS)	INITIAL SERUM Mg (mEq./L.)	ORAL INTAKE (mEq.)	OUTPUT (mEq.)		BALANCE (mEq.)
					URINE	FECES	
R. M.	53	6		109.9	42.2	28.6	+39.0
G. T.	57	9		154.8	32.6	55.8	+66.4
C. F.	61	9	1.36	266.9	49.0	98.7	+119.2
R. E.	61	7	1.52	176.7	54.7	76.6	+45.4
W. L.	89	10	1.36	262.7	27.7	174.6	+60.4
R. H.	89	9	1.48	281.2	17.3	112.2	+151.6
Mean				208.7 (Intake)		128.4 (Excretion)	

Balances of 6 alcoholic patients treated with diet alone are shown in Table IV. The balance periods ranged from 6 to 10 days. The net balances were all markedly positive, ranging from plus 39 to plus 151 mEq.

Table V shows the balance in 5 alcoholic subjects who were given diet plus magnesium sulfate intramuscularly. The net balances were all markedly positive ranging from plus 35 to plus 170 mEq. for the period.

A comparison of the 4 groups of subjects is made in Table VI. The average for control subjects treated with diet alone was a negative balance of 8 mEq., whereas the average for the patients with alcoholism was a positive balance of 80 mEq. Control subjects given diet plus magnesium sulfate injections had a positive balance of 8 mEq., whereas the patients with alcoholism treated similarly had a positive balance of 85 mEq. The positive balance of magnesium was large in patients with alcoholism and tremulousness. When the means of the per kilogram storage of magnesium by the 2 groups of alcoholic patients are compared, it can be seen that there is no essential difference. The net balances of control subjects and patients did not overlap.

Detailed results of the magnesium balance study of Patient R. H. are given in Table VII. The urinary excretion remained low during the 10 days, and it is probable that magnesium repletion was not complete even at the end of this period. The serum magnesium level did not change appreciably. Magnesium repletion probably was not complete in other patients at the completion of the study. It is clear, however, that the patients had a deficit at least as great as the balances indicate.

DISCUSSION

The manifestations of experimentally induced magnesium deficiency in the rat[21] and in the dog[22] include irritability, hyperexcitability, and convulsions. Early cutaneous vasodilatation and late trophic changes of the skin are also seen. Cattle,[23] too, may become magnesium deficient, particularly during spring grazing when the high ammonium content of the fresh green grass grown on land fertilized with nitrates interferes with magnesium absorption from the intestines by forming insoluble magnesium ammonium phosphate in the rumen. The manifestations of deficiency in various species can be corrected by supplying magnesium. More complete discussions of experimental magnesium deficiency are recorded in previous communications.[2, 4, 7]

After withdrawal of alcohol, some patients with severe chronic alcoholism have signs strikingly similar to those of animal magnesium deficiency, including

TABLE V. FIVE ALCOHOLIC PATIENTS TREATED WITH DIET PLUS $MgSO_4$

PATIENT	WEIGHT (KG.)	DURATION OF STUDY	INITIAL SERUM Mg (mEq./L.)	INTAKE (mEq.)			OUTPUT (mEq.)		BALANCE (mEq.)
				ORAL		I.M. $MgSO_4$	URINE	FECES	
				MEASURED	CALCULATED				
D. E.	80	6		94.1	5.0	76.0	66.2	34.7	+74.2
G. S.	55	6	1.58	69.7	71.4	80.0	84.4	23.2	+91.0
W. E.	61	6	1.96	49.0	61.2	48.0	66.5	56.0	+35.7
P. N.	92	6	1.70	66.1	61.5	60.0	59.5	72.9	+55.1
P. T.	88	8	1.66	128.8	69.2	222.0	152.7	96.5	+170.7
Mean				227.9 (Intake)			142.5 (Excretion)		

TABLE VI. COMPARISON OF NET BALANCES OF ALCOHOLIC SUBJECTS AND NONALCOHOLIC SUBJECTS (ALL IN MEAN VALUES, mEq.)

	NONALCOHOLIC CONTROL SUBJECTS	ALCOHOLIC SUBJECTS	
		TOTAL	mEq. PER KG.
Diet alone	−8.3	+80.3	+1.16
Diet plus $MgSO_4$	+7.4	+85.3	+1.14

TABLE VII. PATIENT R. H. TREATED WITH DIET ALONE

DATE	SERUM MG	URINE MG (mEq.)	STOOL MG (mEq.)	INTAKE MG (mEq.)
7/28-7/29				
(12 hr.)		0.72		00.0
7/29-7/30		3.78	14.0	30.2
7/30-7/31	1.48	1.58		30.2
7/31-8/1		1.59	40.6	29.9
8/1-8/2	1.36	2.58		32.4
8/2-8/3		1.02		31.7
8/3-8/4		2.41		31.7
8/4-8/5	1.48	.77	28.8	31.7
8/5-8/6		1.08		31.7
8/6-8/7		1.75	28.8	31.7
Total		17.28	112.2	281.2
Net balance:	151.6 mEq. (16.8 mEq. Mg per day)			

tremor, hyperexcitability, mental disturbances, and convulsions. The serum magnesium may be normal in the patient following chronic overindulgence in alcohol and thus inaccurately reflect changes in the total body stores. Martin and her co-workers[9] have analyzed the electrolyte pattern at time of entry of a group of such patients and found that 60 per cent have low serum magnesium concentrations. Our results show strikingly positive magnesium balances during treatment with diet alone or diet plus parenteral magnesium sulfate in patients after withdrawal from alcohol. These studies confirm our earlier partial balance studies carried out on 3 patients.[7] Control subjects, whether given parenteral magnesium or not, were in balance with respect to magnesium.

The causes of the magnesium deficiency are not clear, although poor oral intake is important. Sufficient calories are supplied by the alcohol, but multiple deficiencies in electrolytes, vitamins, and protein characterize the diet of chronic alcoholics. The kidneys are fairly efficient in conserving magnesium, as seen in Table VII. Even in severe depletion, excretion of magnesium continues at the rate of about 1 to 3 mEq. per day.[24] In addition, evidence has been obtained in limited studies that ethyl alcohol itself may cause increased magnesium excretion.[25]

In view of the evidence that a strongly positive balance occurs from diet alone, it could be concluded that parenteral magnesium is not necessary. This is true of the mildly to moderately ill patients who are able to eat. Animals with experimentally induced magnesium deficiency, likewise, can recover by eating a normal diet, unless they have become very ill. The patients unable to eat any food need parenterally administered magnesium to correct their deficit until they can eat a normal diet.

SUMMARY

Magnesium balance studies were undertaken in 11 patients with chronic alcoholism during 6 to 10 day periods. In comparison with control patients, all alcoholic subjects studied showed markedly positive magnesium balances during the repletion period. The data clearly demonstrate total body magnesium deficiency and underline the need for magnesium repletion, either by diet, parenteral injection, or both, in patients with chronic alcoholism with tremor and hyperkinesis. The more severely ill patients, especially those who cannot eat normally, need parenteral magnesium injections.

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BONE MARROW TRANSPLANT IN ACQUIRED HYPOGAMMAGLOBULINEMIA

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IT IS possible to endow the hypogammaglobulinemic patient with a miniature antibody-producing system by homotransplantation of lymph nodes. Such grafts may function for several months but eventually undergo dissolution.[1, 2] This contrasts with the survival for several years of skin homografts in hypogammaglobulinemic children. The failure of lymph nodes to survive more than a few months may be related to their high capacity for antibody production.

It was felt that a tissue with antibody-producing capacity somewhat less vigorous than that of lymph nodes might survive, as skin grafts have, and still enhance the capacity of the host to form antibody. Bone marrow contains all the cellular elements implicated in antibody synthesis,[3] and, although antibody production is not an important function of marrow, it has been shown to occur after antigenic stimulation.[4] Marrow has the advantages of being relatively easy to obtain and administer.

The bone marrow of hypogammaglobulinemic patients is deficient in that it is devoid of plasma cells, and plasma cell formation which is seen in normal marrow after antigenic stimulation fails to occur.[5]

METHODS

The recipient was a 55-year-old woman with acquired hypogammaglobulinemia, who has been reported in detail.[6] For 2 years after the diagnosis was made she had remained essentially free of infection on a regimen of monthly injections of gamma globulin* and oral sulfonamide.

The donor was the patient's husband, and was normal in so far as could be determined by history, physical examination, and studies of the blood and bone marrow. Both recipient and donor were of identical blood type, i.e., type O, D(+), C(+), E(−), c(−), and e(+).

Bone marrow was obtained from each posterior ilium of the donor by Bierman biopsy.[7] The number of nucleated cells obtained by this method ranges from 1 to 2 billion cells.[8] The marrow fluid, totaling 70 ml., was placed in a plastic bag which contained 5,000 units of heparin in 100 ml. Tyrode's solution, and was immediately administered intravenously to the recipient. Materials in contact with the cell suspension were either made of plastic or were siliconized. No clinical symptoms accompanied the infusion.

The recipient returned at approximately monthly intervals for blood and bone marrow studies. She was challenged antigenically, 6 and 13 days after the transplant, with vaccine containing in each milliliter 1,000 million *Salmonella typhosa* bacilli, 250 million *Salmonella paratyphi*, and 250 million *Salmonella schottmülleri*.† A complete blood count was done at

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†Typhoid-paratyphoid Vaccine, Eli Lilly & Co., Indianapolis, Ind.

each examination. Antibody response was measured by the slide agglutination method devised by Welch and Stuart.[9] Gamma globulin levels were measured by filter paper electrophoresis according to the method of Henry and co-workers.[10] Bone marrow plasma cell counts were made according to the method of Good and Campbell.[11] Serum complement was determined by the method of Wedgewood and Janeway.[12*]

RESULTS

Blood counts remained essentially unchanged except for the leukocyte differential, in which the eosinophils increased from zero (in multiple counts during 2 years before transplant) to 9 per cent 1 month after transplant, with a gradual fall thereafter.

TABLE I. INITIAL SERUM PROTEIN ELECTROPHORESIS RESULTS

DATE	TOTAL PROTEIN	ALBUMIN	GLOBULINS				
			TOTAL	a_1	a_2	b	g
4-11-58	6.1	3.5	2.7	.5	.8	1.1	.3
5-10-58	6.7	3.9	2.8	.5	.8	1.1	.4
6-14-58	7.0	4.3	2.7	.4	.9	1.0	.4
8-18-58	6.9	3.8	3.1	.5	1.0	1.1	.5
9-16-58	6.6	3.7	2.8	.5	.9	1.0	.4
9-24-58	6.9	4.0	2.8	.4	.9	.9	.6
10-10-58	6.3	4.0	2.4	.5	.7	.9	.3
Normal range (Gm. %)	6.2 to 8.5	3.5 to 5.5		0.2 to 0.4	0.5 to 0.9	0.6 to 1.1	0.7 to 1.7

Monthly electrophoretic serum protein analyses indicated a rise in gamma globulin concentrations which reached a level of 0.5 Gm. per cent by the fourth month following the transplant (Table I). The patient's regular prophylactic injections of gamma globulin were then suspended, and 5 weeks later the gamma globulin concentration reached a level of 0.6 Gm. per cent, double that which had been present prior to the transplant. One month later a sharp drop was observed, and it was felt unsafe to withhold the globulin injections any longer.

Serum complement, initially higher than normal, showed an abrupt fall to a very low level, which was followed by a gradual rise during the next 6 months to pretransplant level (Table II).

TABLE II. SERUM COMPLEMENT: 50 PER CENT HEMOLYTIC UNITS PER MILLILITER SERUM

DATE	COMPLEMENT CONCENTRATION (NORMAL RANGE: 25 TO 45)
March 12	69.0 (pretransplant)
April 12	5.0
June 14	7.4
Aug. 18	29.8
Sept. 24	43.0
Oct. 10	60.0

Agglutination titers for typhoid and paratyphoid A and B groups of Salmonella remained at zero level throughout the posttransplant period.

Serial bone marrow plasma cell counts showed no increase in plasma cell population, since only 2 plasma cells were found in the many thousands of nucleated cells counted.

*Through the cooperation of H. M. Kurtz, Ph.D.

Since there was a discrepancy between the electrophoretic results, which seemed to indicate increased gamma globulin production, and the other parameters of immunologic response, additional studies were made.

Aliquots of the monthly serum samples, stored in the frozen state, were checked with a latex fixation gamma globulin test.* No significant variation could be found, all the agglutinations falling in the hypogammaglobulinemic range (50 to 200 mg. of gamma globulin) (Table II). The stored serum samples were then subjected to electrophoresis in a simultaneous run, in order to circumvent some of the variables encountered in separate electrophoretic runs. The results are shown in Table III. No significant change in gamma globulin concentrations was detected. The results of these check studies clearly demonstrated that the initial electrophoretic results were misleading. The indicated rise in gamma globulin concentration was probably due to a concatenation of some of the variables intrinsic in paper electrophoresis.[10] The large range of error, particularly in the lower range of gamma globulin concentrations, has provoked the development of more precise methods of quantitating small amounts of gamma globulin.[13]

TABLE III. GAMMA GLOBULIN VALUES OBTAINED WITH COMPARATIVE TESTS

	ELECTROPHORESIS		
DATE	INITIAL MONTHLY RUNS (GM. %)	SIMULTANEOUS RERUN	LATEX FIXATION TEST SIMULTANEOUS RUN
April, 1958	0.3	0.4	PNN
May, 1958	0.4	0.5	PNN
June, 1958	0.4	0.5	PNN
August, 1958	0.5	0.5	PNN
Sept., 1958	0.4	0.4	PNN
Oct., 1958	0.6	0.4	PNN
Jan., 1959	0.4	0.4	PNN

Code: NNN = 25 mg. %
PNN = 50 to 200 mg. %
PPN = 600 to 1,200 mg. %
PPP = 3 to 5 Gm. %

DISCUSSION

The fact that no truly significant change in gamma globulin level occurred is not surprising, since no rise in serum gamma globulin concentration was detected in previously reported agammaglobulinemic patients with temporarily successful lymph node homotransplants, even though the grafts were able to produce measurable antibody.[1, 2]

In one of these patients morphologic survival of the grafted lymph node cells was demonstrated by serial biopsies.[2] Serial bone marrow aspirations in our patient showed no plasma cell increase, and other methods for study of donor cell survival could not be applied. Had the recipient been male and the donor female, it might have been possible to demonstrate a mosaic pattern of the leukocyte population by quantitative studies of the sex chromatin body in

*Through the cooperation of R. Fisk, Ph.D., Research Division, Hyland Laboratories, Los Angeles, Calif.

granulocytes or monocytes. Search for a mosaic erythrocyte pattern was precluded by the fact that donor and recipient were of identical blood type.

The dichotomy in normal immune mechanisms is sharply delineated in agammaglobulinemic patients, because they have lost principally the ability to form circulating antibodies of the gamma globulin type (and occasionally some of the beta globulins). The capacity to produce alpha and beta globulins, and tuberculin-type fixed tissue antibody is largely intact.[14] It is cell-bound antibody which is chiefly responsible for the rejection of grafts.[15]

It has been postulated that acquired agammaglobulinemia may be one of the autoimmune diseases in which the patient produces antibodies to his own antibody-forming system.[16] While this appears paradoxic, there is clinical evidence that agammaglobulinemic patients are capable of producing the factors involved in autoimmune disease. In two large groups of agammaglobulinemic patients, scleroderma, dermatomyositis, and rheumatoid arthritis have occurred with disproportionate frequency, the latter with an incidence approaching 50 per cent.[14, 17]

The rise in the peripheral eosinophil count in our patient suggested an allergic impact of the bone marrow infusion. Severe bronchial asthma accompanied by eosinophilia, in which the eosinophil count was as high as 50 per cent of the peripheral leukocyte count, has been observed in a hypogammaglobulinemic adult.[18] Allergic phenomena in patients deficient in gamma globulin have become more understandable in the light of recent work which indicates that atopic reagins are alpha and beta globulins.[19]

The observed fall in serum complement concentration following marrow transplantation is compatible with complement utilization in the course of an antigen-antibody reaction. The prolonged depression of complement would imply a continuing reaction involving an antigen which was very slowly eliminated. It is known that in systemic lupus erythematosus (S.L.E.) serum complement levels remain low for long periods when the disease is clinically active, and become normal or slightly elevated when the disease goes into remission.[20] Pearson and Craddock[21] have demonstrated complement fixation reaction against purified desoxyribonucleic acid or a lysate of human leukocytes in sera of patients with severe S.L.E. Clinical manifestations of a lupoid reaction were absent in our patient, and blood smears remained negative for L.E. cells.

Although an immune reaction probably occurred, it would appear that our patient was neither helped nor harmed by the bone marrow transplant. Since there are 3,000 hypogammaglobulinemic patients in this country alone,[22] continued search for means of restoring the antibody-producing capacity of these patients seems worth while. It is likely that homografts of tissue with antibody-producing capacity will not succeed unless some means of circumventing the host's rejection of the graft is employed. Brephoplastic grafts[23] and protection of grafts with diffusion chambers are two promising techniques which have not yet been applied in agammaglobulinemia.

Once this problem is solved we may then be faced with the problem of rejection of the host by the graft.

SUMMARY

1. Transplantation of normal bone marrow to a patient with acquired hypogammaglobulinemia was attempted.

2. Morphologic studies failed to demonstrate a change in the plasma cell count of the recipient's marrow.

3. Electrophoretic and agglutination studies showed no true increase in production of gamma globulin or capacity to respond to antigenic challenge with bacterial vaccine.

4. Peripheral blood eosinophilia and prolonged depression of serum complement were observed. The similarity of this reaction to certain aspects of systemic lupus erythematosus is discussed.

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A STUDY OF THE ANTIGENS OF NORMAL LEUKOCYTES

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THE purpose of this article is to report an investigation of the antigens of normal leukocytes.

Recent studies of Brittingham[1] and Payne[2] have indicated that human leukocytes contain isoantigens which stimulate antibody formation through pregnancy and blood transfusion. The identification of leukocytic isoantigens, therefore, presents a problem of some importance. The techniques for the identification of red cell antigens have been very productive, but, for the most part, these methods are not applicable to the study of white cells. The bulk of knowledge concerning the red cell antigens has been gained by testing human red cells for agglutination in human sera. For the most part these serum antibodies are either naturally occurring or are acquired by isoimmunization. Only a small percentage of human sera, however, has been found to contain naturally occurring leukocyte agglutinins.[1, 3, 4] These agglutinins are weak and the methods presently available for their study are not entirely satisfactory. Indeed some authors have been unable to demonstrate leukocyte agglutinins in studying large numbers of normal individuals.[5, 6] There are conflicting reports from several laboratories concerning tests for the ability of serum from patients who were either repeatedly transfused or had other diseases of a hematologic nature to agglutinate the leukocytes from a panel of normal donors. Some authors,[1, 5, 6] have indicated that a high percentage of the leukocyte suspensions were agglutinated. Dausset and associates,[7] and Lalezari and Spaet,[8] were able to detect several patterns of agglutination in testing the white cells of normal individuals with leukocyte-agglutinating sera. More recently Payne and Rolfs[9] have reported isoleukoagglutinins in sera from mothers which react differently with their panel of 10 leukocyte donors so that no 2 sera were identical. The finding that these sera from mothers agglutinated the leukocytes of 10 of 12 of their respective newborn infants, and 12 of 13 of their respective spouses, suggested to these authors that the offspring had inherited the fathers' leukocyte factor.

The M, N, P, and Rh blood groups were discovered by immunizing animals with human red cells and subsequently absorbing this antiserum with red cells possessing all other red cell antigens. The present study adapts this method to an investigation of human leukocyte antigens by immunizing animals with leukocytes from humans and subsequently absorbing this immune serum with leukocytes from different individuals.

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METHODS

Preparation of Antileukocyte Plasma.—One hundred and fifty milliliters of blood was withdrawn from a human donor (immunizing donor) through plastic tubing into a 500 ml. flask containing 15 ml. of 1 per cent sodium ethylenediamine tetra-acetic acid, and 20 ml. of dextran having an intrinsic viscosity greater than 0.41. After allowing the red cells to settle for 30 minutes the supernatant plasma was drawn off and equal amounts were transferred to four 50 ml. centrifuge tubes. Saline was added to the 50 ml. mark and this was centrifuged at 1,000 r.p.m. for 15 minutes. The sediment was washed 3 times with an isotonic solution of saline; it consisted predominantly of leukocytes but was contaminated with small numbers of red cells and platelets. After the final centrifugation the supernatant was discarded and the packed sedimented cells were suspended in 1 ml. of modified Freund's adjuvant.[10] This was prepared by mixing 10.8 ml. of Arlacel A,* 61.2 ml. of Bayol F,† and 50 mg. of dried *Mycobacterium butyricum.* This was injected subcutaneously into a rabbit and the injection was repeated the second and third weeks. A similar injection of leukocytes suspended in saline was given subcutaneously the fifth week. At the end of 6 weeks the rabbits were bled to exsanguination by withdrawal of blood from the aorta through a No. 20 gauge needle which was connected by polyethylene tubing to a 100 ml. Erlenmeyer flask containing ethylenediamine tetra-acetic acid. The plasma was removed immediately and heated to 56° C. for 30 minutes; this procedure caused some precipitation of fibrin which was removed by centrifugation. Ten per cent by volume of a 1 per cent aqueous solution of Merthiolate was added to the plasma and it was stored at -20° C. until used. All glassware and needles were coated with Siliclad,‡ and polyethylene tubing was used. These procedures were carried out under sterile precautions.

Three rabbits were subjected to a mock course of immunization utilizing Freund's adjuvant alone on the first, second, and third weeks, and saline on the fifth week. Blood was drawn before this procedure and again on the sixth week.

Absorptions.—Beef kidney was cut up and emulsified in a Waring Blendor. After centrifugation it was washed with isotonic saline solution until the supernatant was clear. Antileukocyte plasma was mixed with equal amounts of washed beef kidney and incubated at 37° C. for 60 minutes. This absorption with beef kidney was repeated three times on the plasma aliquot.

Absorption with red blood cells was carried out by adding equal parts of thrice washed red cells and antileukocyte plasma and allowing them to incubate at 37° C. for 60 minutes. Each aliquot of plasma was absorbed until the red cell agglutinin was exhausted. This usually occurred after 3 to 6 absorptions.

Leukocytes and platelets that were used for absorption were prepared in a manner similar to that used to prepare the white cells to immunize the rabbits. Five hundred milliliters of blood was drawn into a siliconized vessel containing ethylenediamine tetra-acetic acid and dextran. After settling for about 30 minutes the supernatant plasma was centrifuged at 1,000 r.p.m. for 45 minutes. This sedimented all but a few leukocytes but left the great majority of platelets suspended. The supernatant was decanted and centrifuged at 3,000 r.p.m. for 10 minutes. The platelets were washed once with ethylenediamine tetra-acetic acid and again centrifuged; the white cells were treated similarly. Two milliliter aliquots of immune plasma were absorbed with the platelets and white cells which 500 ml. of whole blood yielded. This usually amounted to about 2 to 3 ml. of platelets and an equal amount of white cells; each included a certain small percentage of admixed red cells. Each platelet and leukocyte absorption was carried out 3 times unless otherwise noted.

Test for Leukocyte Agglutinins.—Blood was collected according to the method previously described.[3] The test was performed by mixing 0.10 ml. of white cell suspensions with 0.10

*Arlacel A was obtained from Ruger Chemical Co., New York City.
†Bayol F was obtained from Esso Refining Co., Linden, N. J.
‡Siliclad was obtained from Clay-Adams, Inc., New York City.

ml. of test plasma in a 10 by 75 mm. serologic test tube. This was incubated in a water bath at 37° C. for 1 hour. The leukocytes were resuspended after incubation by shaking the test tube quickly about 6 times. A drop was poured on a glass slide and allowed to flow out in a thin layer by tilting the slide. The slide was studied for white-cell clumping under the low power of the microscope either with reduced light or with phase microscopy.

Precipitin Test.—The undiluted antileukocyte plasma was placed in capillary tubes and human plasma which was serially diluted with saline was layered over it. These tubes were incubated at 37° C. for 4 hours, and at 4° C. for 12 hours, after which they were observed for precipitated material.

RESULTS

The following rabbit plasmas were tested for the presence of leukocyte agglutinins against a panel of 6 donors, at least one of whom was of blood group O, A, or B: (1) plasma from 2 rabbits which had received no injections, (2) plasma from 2 rabbits which had received a mock immunization with Freund's adjuvant, and (3) plasma from 3 rabbits one of which had been immunized with leukocyte suspensions from a donor of blood groups O+, one from a donor of group A+, and one from a donor of group B+. Aliquots of this plasma were tested before and after absorption with each of the following: Beef kidney, red cells, white cells, and platelets from the immunizing donor.

The results were essentially the same for the leukocytes of all donors tested. There was minimal leukocyte agglutination in the undiluted specimen of plasma which was taken before and after the course of injections of Freund's adjuvant alone. The average titer of leukocyte agglutinins in the unabsorbed plasma taken after the rabbit had been immunized with leukocytes was 1 to 4,096. This titer was unaffected by absorption of the antinormal leukocyte plasma with beef kidney or red cells, and platelets of the immunizing donor. After absorption of this plasma with leukocytes from the same donor, on the other hand, the tests for leukocyte agglutination were negative.

Anti Red and White Cell Agglutinin.—It was noted that when the unabsorbed immune plasma was tested for leukocyte agglutinins a mixed red- and white-cell clump occurred in dilutions through 1 to 64. This was not present after absorption of the plasma with the immunizing donor's red cells or Type O red cells. The mixed red- and white-cell clumps were not composed of erythrophagocytes.

The plasma of two rabbits, each of which had been immunized with the leukocyte suspensions of different Type A donors, was tested for red cell agglutination against cells of Types O, A, and B. Each plasma produced agglutination to titers of 1 to 32 against cells of Types O and B, and of 1 to 256 against Type A cells. Aliquots of this plasma were absorbed with red cells of Type O. When subsequently tested against red cells of Types O and B there was no agglutination; against red cells of Type A the titer was 1 to 64.

Absorption of Antinormal Leukocyte Plasma by Homologous Leukocytes.—Groups, each comprised of 4 rabbits, were immunized with the leukocytes suspension of a different normal human donor. Sixteen 2 ml. aliquots of immune plasma from the 4 rabbits were subjected to the following absorptions: (1)

each aliquot was absorbed with the red cells of the immunizing donor; (2) one aliquot of plasma from each rabbit was absorbed on 3 different occasions with the leukocytes from 500 ml. of whole blood of the immunizing donor; and (3) each of the other 12 aliquots was absorbed on 3 different occasions with the leukocytes from 500 ml. of blood from one of 12 donors. (Each aliquot was absorbed with leukocytes from only one donor.)

These absorbed plasmas were tested for leukocyte agglutination against the white cells of a panel of 17 normal donors. A total of 159 tests for leukocyte agglutinins was performed on these absorbed immune plasmas; 42 of these were control tests for adequacy of absorption. Of the remaining 117 tests there were 6 which contained agglutinins for other leukocytes. The positive tests for leukocyte agglutination were present in the undiluted sample only. These results are listed in Fig. 1.

PLASMA: IMMUNE TO CELLS OF DONOR NO.	ABSORBED WITH CELLS OF DONOR NO.	TEST CELLS A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	CONTROL
1	1	0	0	0	0	0	0	0	0	0	0	0							
1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	6	0	0	0	0	0	0	0											0
1	7	+	0	0	0	+	0	0	0	0	+	0	0	0	0	0	0	0	
2	2	0	0	0	0	0	0	0											
2	8	0	0	0	0	0	0	0	0	0	0	0	0	0					
2	9	0	0	0	0	0	0	0											0
2	10	0	0	0	0	0	0	0	0	0	0	0	0						0
3	3	0	0	0	0	0	0												
3	11	+	0	+	0	0	0												0
3	12	+	0	0	0	0	0	0	0				0						
4	4	0	0	0	0	0	0	0											
4	13	0	0	0	0	0	0	0	0	0									0
4	14	0	0	0	0	0	0	0	0	0	0	0							0
4	15	0	0	0	0	0	0	0											0
4	16	0	0	0	0	0	0	0											0

Fig. 1.—This figure summarizes the results of tests of rabbit antihuman leukocyte plasma (absorbed with homologous leukocytes) to agglutinate the leukocytes of a panel of normal donors. The arabic numerals in the 2 vertical columns refer to different leukocyte donors. The leukocytes from donors in the first column were used to immunize the rabbits. The leukocytes from donors in the second column were used to absorb the immune plasma. The letters of the alphabet refer to donors whose leukocytes were used in testing for agglutinins. The heavy lines indicate a control test; heavy lines enclosing a single result indicate that the cells tested are the same as those used to absorb the immune plasma. The 0 designates a negative test for leukocyte agglutination and the + a positive test.

Precipitin tests against normal human plasma, performed on plasma from the 4 rabbits which were immunized with leukocytes from a normal donor, were negative.

DISCUSSION

Results of the use of absorbed immune heterologous serum to detect antigens must be interpreted with a certain amount of caution. It is possible, for example, that isoantigens were present on the leukocytes used to immunize the rabbits which did not stimulate antibody production. On the other hand, it is possible that nonspecific substances adherent to the surface, or foreign material phagocytized by the leukocyte, might stimulate antibody formation. The

negative precipitin tests between antihuman leucocyte plasma and normal plasma do not preclude the possibility that antihuman plasma was present in amounts too small to be detected by this method. Another disadvantage of the method lies in the fact that heterologous immune serum contains lysins which are difficult or impossible to remove by absorption. These antibodies may interfere with the results if a method of testing is used which might detect them. Complement is required for the detection of hemolysins and by inference for leukolysins. In the methods used in the present study, however, there is little or no complement activity because of the presence of ethylenediamine tetraacetic acid which binds the divalent cations. The end point in this method is agglutination which does not require complement. Leukocyte agglutination is a rather insensitive method for this reason; as has been discussed previously[3] it was chosen for this purpose to avoid false positive reactions.

It may be that this feature is an advantage when used to study absorbed heterologous immune serum. At any rate the negative controls were completely free of leukocyte agglutinins.

The failure of the various absorbents to alter the titer of the immune rabbit plasma for leukocytes indicates that the leukocytic antigens under study are not related to these substances. The fact that the immunizing donors' red cells and platelets failed to alter this titer demonstrates that these leukocytic antigens are unrelated to those of the red cell or platelet. The lack of influence of the absorption with beef kidney rules out a heterophile antigen of the Forssman variety. There is an apparently nonspecific leukocyte agglutinin in normal rabbit plasma, but this is only present in the undiluted specimen, and it is not increased in the control animals subjected to a mock course of Freund's adjuvant. The leukocyte agglutinins present in the immune rabbit plasma before absorption with leukocytes are active against all white cell suspensions tested. This implies that there is an antigen(s) which is shared by all human leukocytes. Appropriate absorptions with other human tissues were not carried out so that this could be designated an organ-specific antigen. The agglutinin which caused mixed red- and white-cell clumps is apparently stimulated by an antigen(s) which is shared by red and white cells. Again the proper absorptions with other human tissues were not done to prove this is a species-specific effect. The mixed red- and white-cell clumps were not associated with erythrophagocytosis which was the case in other circumstances.[3] The immune rabbit plasma used in experiments to detect leukocytic isoantigens was absorbed with immunizing donors' red cells to remove the antigen shared by the red and white cells.

The positive test for leukocyte agglutinins performed with immune rabbit plasma after absorption with immunizing donor's red cells and homologous leukocytes is interpreted as probable evidence of isoantigenic differences in human leukocytes. Further absorptions of the immune plasmas to determine similarity or differences in the antigens these represent would have been desirable. It should be appreciated, however, that the very small yield of immune plasma precluded any further experiments.

SUMMARY AND CONCLUSION

Antihuman leukocyte rabbit plasma was prepared which contained an agglutinin for all normal human leukocytes tested. This agglutinin was unaffected by absorption with red cells and platelets of the donor whose leukocytes were used to immunize the rabbit (immunizing donor). This immune rabbit plasma also contained an agglutinin associated with an antigen common to both red and white cells.

Sixteen aliquots of 4 different antihuman normal leukocyte rabbit plasmas were exhaustively absorbed with immunizing donors' red cells and the leukocytes from 16 donors, 4 of whom were the immunizing donors. Each aliquot was absorbed with the leukocytes from 1 donor. These aliquots were tested against the leukocytes of a panel of 17 normal donors. A total of 159 tests for leukocyte agglutination was performed of which 42 were controls. Six of the remaining 117 tests were positive for leukocyte agglutinins. This represents probable evidence for the presence of isoantigens in human leukocytes.

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IN VITRO EFFECTS OF ANTIBIOTICS ON YEAST PHASE OF BLASTOMYCES DERMATITIDIS AND OTHER FUNGI

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MEDIA containing antibiotics for the selective isolation of pathogenic fungi are currently being employed by many laboratories throughout the world.[1-6] Through the inhibition of bacteria and saprophytic fungi, great advantages have been realized in the isolation of pathogenic fungi from diverse substrata. However, there are certain limitations to the use of such selective media, and these should be well recognized and understood. Recent studies have indicated that temperature of incubation influences the sensitivity of certain pathogenic fungi to several of the antibiotics currently used in such selective media.

While testing the effects of antibiotics on *Blastomyces dermatitidis* it was observed that mycelial transfers of 21 strains on media containing 0.5 mg. per milliliter cycloheximide and 0.05 mg. per milliliter chloramphenicol grew well at room temperature. On the other hand, duplicate tubes of medium, when inoculated with yeast cells of this fungus and incubated at 37° C., failed to support growth or permitted only minimal development. Since the use of media containing cycloheximide and chloramphenicol has become a common practice in medical mycology this observation seemed worthy of further study.

MATERIALS AND METHODS

Thorough testing of the effects of temperature on sensitivity was accomplished as follows: serial dilutions (Table I) of antibiotic in brain heart infusion agar were used.

Table I. Effects of Cycloheximide on Growth of Blastomyces Dermatitidis After 3 Weeks at 37° C. on Brain Heart Infusion Agar

Antibiotic concentration (milligram per milliliter)	Strains tested								
	A	B	C	D	E	F	G	H	I
2.0	3	−	+	+	−	−	−	−	−
1.0	4	1	1	1	+	−	−	−	−
0.8	4	2	2	2	+	−	+	−	−
0.6	4	3	2	2	1	+	+	−	−
0.4	4	3	3	3	2	+	+	−	−
0.2	4	3	3	3	3	1	1	−	−
0.1	4	4	3	3	3	2	2	1	+
0.05	4	4	4	3	4	3	3	2	+
0.025	4	4	4	4	4	3	3	4	2
Control	4	4	4	4	4	4	4	4	4

Growth in control tubes containing no antibiotic was considered as 4 at time of each reading. + stands for trace.

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Suspensions of yeast cells of 9 strains of the fungus in physiologic salt solution were prepared so that each milliliter contained approximately 600,00 yeast cells. One one-hundredth milliliter of the suspension was streaked on the surface of the agar slant in each culture tube. Beginning on the seventh day tubes were examined at weekly intervals until 3 recordings had been made.

RESULTS

There is great variation among the different strains of *B. dermatitidis* in capacity to grow on B. H. I. cycloheximide agar at 37° C. (Table I). Thus after 3 weeks on this medium, strain A was not inhibited except at a concentration of 2.0 mg. per milliliter. On the other hand, strain I produced only a trace of growth in tubes containing 0.1 mg. per milliliter, and no growth in tubes containing greater concentrations. Since concentrations of 0.5 or 0.4 mg. are commonly used in medical mycology in media designed for isolation of pathogenic fungi from clinical materials, strains F, G, H, and I would probably not have been recovered if the isolation tubes had been incubated at 37° C. No doubt the number of cells in the inoculum has an effect on survival and growth of the organism in the presence of the antibiotic. In a similar experiment, but using one half the number of yeast cells per milliliter, it was observed that only strain A grew on medium containing 0.5 mg. per milliliter cycloheximide.

To verify that some strains of *B. dermatitidis* are more sensitive to cycloheximide in the yeast phase than in the mycelial phase 9 strains were tested simultaneously at 25° C. and at 37° C. Table II shows results obtained with 3 isolates when yeast cells were used as inocula. The results indicate that all strains tested showed sensitivity at both 25° C. and at 37° C. The inhibition was greater, however, in all cases during the early stages of growth at 37° C.

TABLE II. EFFECTS OF TEMPERATURE ON GROWTH OF BLASTOMYCES DERMATITIDIS ON B. H. I. CYCLOHEXIMIDE AGAR

ANTIBIOTIC CONCENTRATION (MILLIGRAM PER MILLILITER)	STRAINS TESTED AND WEEKS OF GROWTH								
	A			C			H		
	1	2	3	1	2	3	1	2	3
	Growth at 25° C.								
2.0	+	2	3	1	2	3	+	3	3
1.0	1	3	4	2	3	3	1	4	4
0.8	1	3	4	2	3	3	1	4	4
0.6	1	3	4	3	3	4	1	4	4
0.4	2	3	4	3	3	4	2	4	4
0.2	3	3	4	3	4	4	2	4	4
0.1	3	4	4	3	4	4	2	4	4
0.05	3	4	4	3	4	4	2	4	4
0.025	3	4	4	3	4	4	3	4	4
Control	4	4	4	4	4	4	4	4	4
	Growth at 37° C.								
2.0	−	+	3	−	−	+	−	−	−
1.0	−	1	4	−	−	1	−	−	−
0.8	+	1	4	−	+	2	−	−	−
0.6	+	2	4	−	+	2	−	−	−
0.4	+	3	4	−	+	3	−	−	−
0.2	+	3	4	−	+	3	−	−	−
0.1	+	3	4	+	2	3	−	−	1
0.05	1	4	4	2	3	4	−	+	1
0.025	2	4	4	2	3	4	−	1	4
Control	4	4	4	4	4	4	4	4	4

as compared with early stages at 25° C. It was noticeable that at 37° C. only one of the 3 strains (A) produced observable growth after 7 days in tubes containing 0.4 mg. per milliliter. Of the 9 strains studied, only one other isolate was as resistant as A during early growth at 37° C. On the other hand, all of the strains produced as much growth on 0.5 mg. per milliliter cycloheximide media at 25° C. by the end of the third week as they produced on antibiotic-free media. Therefore significant inhibition by 0.5 mg. per milliliter cycloheximide media of the growth of these strains of *B. dermatitidis* was associated with incubation at 37° C. and not with incubation at 25° C.

Other experiments demonstrated that the constituents of the test media, other than the antibiotics, had no effect on the 25° to 37° sensitivity ratio. Brain heart infusion broth, Sabouraud's dextrose agar, and Sabouraud's dextrose broth revealed the same general resistance pattern. The use of conidia rather than yeast cells as an inoculum also did not alter this ratio.

Experiments similar to those described with cycloheximide were performed with chloramphenicol at concentrations of 0.2, 0.1, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, and 0.0025 mg. per milliliter of medium. Increased sensitivity at 37° C. was observed. In general, the isolates so far studied have been less sensitive to chloramphenicol at concentrations commonly used in isolation media (0.05 mg. per milliliter) than to cycloheximide at 0.5 mg. per milliliter. At least 2 strains, however, have shown such reduced growth at 0.05 mg. per milliliter as to make it unlikely that they would be isolated on media containing this quantity of chloramphenicol at 37° C.

From the practical viewpoint and also in regard to the general question of the cause of the mycelial-yeast sensitivity ratio it was thought to be important to know the effect of these antibiotics on other fungi. For this reason *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, and *Sporotrichum schenckii* were grown on antibiotic media at 25° C. and 37° C. as had been done for *B. dermatitidis*. It has not been possible to detect a definite 25° to 37° C. sensitivity differential with the few strains of *P. brasiliensis* so far studied. Such a differential exists for some strains of *H. capsulatum* and *S. schenckii*, but to a lesser degree than for *B. dermatitidis*.

DISCUSSION

The fact that *B. dermatitidis* has been reported[7] to be resistant to cycloheximide at a concentration of 0.5 mg. per milliliter was undoubtedly due to the fact that the tests were carried out at room temperature.

Cycloheximide medium was originally developed to facilitate the isolation of human pathogenic fungi from clinical materials and substrata heavily contaminated with both bacteria and saprophytic fungi.[7] This medium has proved to be highly efficient and adaptable for many types of investigations.[1-6] The observation that some strains of *B. dermatitidis* are inhibited by cycloheximide at 37° C. indicates that in routine diagnostic procedure, media with and without cycloheximide must be used in parallel, particularly if incubation is carried out at 37° C.

SUMMARY

Nine strains of *Blastomyces dermatitidis* showed more sensitivity to cycloheximide at 37° C. than at 25° C. At 37° C., 2 isolates failed to grow on media containing 0.4 mg. per milliliter cycloheximide. Chloramphenicol had a lesser effect, but a similar one. It has not been possible to detect a definite 25° C. to 37° C. sensitivity differential with the few strains of *Paracoccidioides brasiliensis* so far studied. Such a differential exists for some strains of *Histoplasma capsulatum* and *Sporotrichum schenckii*, but to a lesser degree than for *B. dermatitidis*.

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TELLURITE REDUCTION AS AN INDICATOR OF POTENTIALLY PATHOGENIC STAPHYLOCOCCI

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WHEN culture of bacteria on artificial media became technically feasible, staphylococci were among the first bacteria grown. Rosenbach's monography of 1884,[1] in establishing staphylococcus as a distinct bacterial genus, provided a taxonomic basis for demonstration of the widespread occurrence of staphylococcus. This very ubiquity of staphylococcus raised the problem of differentiating those staphylococci which can cause disease from those which ordinarily do not cause disease.

Many laboratory criteria have been advocated as accurate indicators of disease-producing potential in staphylococcus. In using the terms *aureus* and *albus* for species designation, Rosenbach gave prominence to pigment production as a property of virulent staphylococci. Hemolysis, mannitol, fermentation, plasma coagulation, protease production, and tolerance to a hyperosmolar environment have also been noted as biochemical accomplishments displayed by many potentially pathogenic staphylococci.

That some staphylococci were capable of reducing tellurite, was probably first appreciated by workers using tellurite-containing media for isolation of *Corynebacterium diphtheriae*. Jet black colonies on a medium such as that described by Mueller and Miller,[2] must always be examined by Gram-stained smear since some staphylococci, as well as *mitis* strain *C. diphtheriae*, grow in colonies of this appearance.

Use of tellurite as a selective agent for isolation of pathogenic staphylococci was apparently first suggested by Ludlam,[3] in 1949. Ludlam's medium proved too inhibitory for use in primary isolation of staphylococci.[4, 5]

From Ludlam's formula, Zebovitz and associates[6] derived a tellurite medium for selective culture of staphylococcus. The medium was developed by tests performed with 22 laboratory strains of staphylococcus (10 coagulase-positive; 12 coagulase-negative). After trial with a few samples of air, dust, soil, food products, feces, and cultures of skin, nose, and throat, these authors recommended their tellurite-glycine agar for *detection* of coagulase-positive staphylococci. To our knowledge, this tellurite-glycine agar medium has not been evaluated with regard to its utility in the *differentiation* of potentially

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pathogenic and nonpathogenic staphylococci obtained in cultures of clinical specimens. Accordingly, we assessed the differential value of the tellurite-glycine medium of Zebovitz and associates,[6] using 251 isolates of staphylococcus from clinical materials and 73 isolates from the antecubital skin of normal, healthy adults. Ability to grow and reduce tellurite on this medium was interpreted in terms of simultaneously determined ability to coagulate plasma, ferment mannitol, lyse sheep erythrocytes, grow in hypertonic media, and hydrolyze gelatin. These data were not accumulated simply to aggrandize further the enzymatic prowess of staphylococcus. Rather, the aim of this study was the development of a laboratory test for potential pathogenicity of staphylococcal isolates which would be at least as reliable and informative as presently employed tests—a test which, at the same time, would be easier to carry out. Assay for tellurite reduction fulfills these requirements.

MATERIALS AND METHODS

Staphylococcal Isolates.—Two hundred and fifty-one strains of staphylococcus were collected as isolated from cultures of clinical specimens submitted to the Clinical Microbiology Laboratory of the Salt Lake County General Hospital. Identified only by specimen number, these isolates were subcultured on sheep blood agar plates which were sealed and stored at 4° C. For purposes of comparison, additional cultures of staphylococcus were obtained by swabbing the skin of the antecubital space of normal, healthy adults over the usual site for venipuncture. Seventy-three isolates of staphylococcus were collected in this manner from hospital personnel—physicians, nurses, secretaries, and technicians.

For testing, all isolates were inoculated into 2 ml. (13 × 100 mm. tubes; aluminum caps) of simple nutrient broth containing no fermentable carbohydrate.[7] After overnight incubation at 37° C., growth was suspended before inoculation (3 mm. loopful) of eighth sectors of each of 4 kinds of medium: sheep blood agar, mannitol-chalk agar, Chapman-Stone agar, and tellurite-glycine agar. The remaining bacterial cells were then sedimented by centrifugation; the supernatant was sucked off and discarded in preparation for coagulase testing.

Coagulase Test.—The button of sedimented staphylococcal cells was suspended in 0.5 ml. of freshly reconstituted, commercial, dehydrated plasma.* A known coagulase-positive staphylococcus and an uninoculated broth control, both treated in the same fashion as the test isolates, were included. All tubes were placed in a water bath at 37° C. Readings were carried out by removing each tube from the bath, tilting to observe for clotting, at one-half, 1, 2, 4, and 6 hours after the test was set up. The tubes were left at room temperature after the 6 hour reading; further observations were made when 18 and 24 hours had elapsed after inoculation. Any degree of clotting was taken as a positive coagulase test.

Tellurite Reduction.—Commercially available tellurite-glycine agar† was prepared according to the manufacturer's instructions. After inoculation, the inverted plates were incubated overnight at 37° C. Growth in jet black colonies in this period of time (18 hours) was accepted as tellurite reduction.

Mannitol Fermentation.—The medium of Korman and Berman[8] was used to test for mannitol fermentation by overnight incubation at 37° C., in a candle-jar. Isolates capable of fermenting mannitol grew as yellow colonies, vivid against the blue background color of the medium (bromthymol blue indicator).

Hemolysis.—Sheep blood agar plates (2 per cent citrated sheep blood added to commercial tryptic digest of casein-papaic digest of soy bean agar base) were inoculated as described. Plates were inspected for hemolysis after overnight incubation at 37° C., in a candle-jar.

* Bacto-coagulase plasma, Difco Laboratories, Inc., Detroit, Mich.
†Tellurite-glycine agar, Baltimore Biological Laboratories, Baltimore, Md.

Hypertonic Environment, Pigment, and Gelatinase.—Chapman-Stone medium, containing 5 per cent sodium chloride, 7.5 per cent ammonium sulfate, and 3 per cent gelatin was prepared according to Chapman's formulation.[9] All 251 clinical isolates, as well as the 73 skin isolates of staphylococcus, grew on this medium. After 48 hour incubation at 37° C., the presence of any shade of color, cream through orange, was interpreted as an indication of pigment production. Those isolates capable of hydrolyzing gelatin rendered the surrounding medium transparent.

Pathogenicity.—Hospital records of the patients from whom the 251 isolates from clinical specimens had been obtained were examined. Decision as to whether or not a particular isolate was actually the cause of disease could not be made in every case. However, 32 isolates from pus obtained on drainage of purulent lesions were judged to be indubitable pathogens. Fifty-four isolates from the nose or throat were classed as definite nonpathogens. Fifteen isolates from blood cultures were considered separately; a blood culture isolate was classed as a pathogen if it was one of a series of several positive cultures obtained from a given patient, and if the clinical course of this same patient was consistent with staphylococcal sepsis.

Virulence.—Four clinical isolates and 7 staphylococcal isolates from the antecubital skin of normal adults were tested for virulence in rabbits. Eighteen-hour-old broth cultures, in tryptic digest of casein-papaic digest of soybean broth, were harvested by centrifugation at 4° C. The collected cells were washed 2 times with 0.9 per cent sodium chloride solution containing 0.01 per cent Tween 80. After a third washing with plain 0.9 per cent sodium chloride solution, the staphylococci were suspended in a volume of 0.9 per cent sodium chloride solution equal to 1 per cent of the original culture volume. After direct counting in the Petroff-Hauser chamber, dilutions were prepared in 0.9 per cent sodium chloride, which gave in each 0.1 ml. numbers of staphylococci in the ranges: 10^{10}, 10^{9}, 10^{8}, and 10^{7}. Each dilution of each isolate tested was injected intradermally into the previously shaved skin of a full grown albino rabbit. The sites of inoculation were observed daily for the succeeding 14 days. The smallest inoculum which evolved into a lesion which discharged pus was taken as the minimal intradermal infecting dose for that isolate.

RESULTS

In general, the clinical specimen isolates of staphylococcus were far more reactive than were staphylococci isolated from the skin of normal subjects (Fig. 1). In both groups, tellurite reduction, plasma coagulation, and pigment production were in agreement.

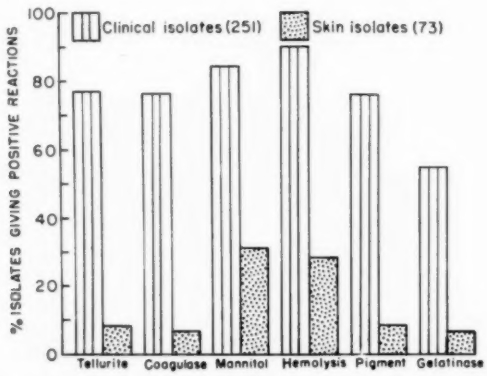

Fig. 1.—The per cent of 251 clinical specimen isolates of staphylococcus which reduced tellurite, coagulated plasma, fermented mannitol, lysed sheep erythrocytes, elaborated pigment, and lysed gelatin on the Chapman-Stone medium, is contrasted with the per cent of 73 antecubital skin (normals) isolates of staphylococcus which also accomplished these reactions.

The data regarding the clinical specimen staphylococci which were surely pathogenic (32 from pus) and surely nonpathogenic (54 from the upper respiratory tract) in the patients from whom they were isolated are presented in Table I together with the skin isolate findings. Again, tellurite reduction, plasma coagulation, and pigment production were consistent parameters. Furthermore, while pathogen was not clearly differentiated from nonpathogen among clinical specimen isolates, skin staphylococci were definitely less reactive by these tests than either kind of clinical specimen staphylococcus. The results of the intradermal virulence testing of the 11 isolates previously described (Table II) confirmed that greater capacity to become established in tissues and cause disease is another attribute of the biochemically accomplished staphylococcus.

Fifteen blood culture isolates were included in the 251 clinical isolates. Of these, on clinical grounds 7 were found to be responsible for illness and 8 were not. Tellurite, coagulase, and mannitol reactions were the most accurate indicators of the reactions which were tested (Table III).

The tube coagulase test is generally accepted as being the best single laboratory indicator of potential pathogenicity of staphylococcus.[10] The rate at which 191 of the 251 isolates from clinical specimens coagulated plasma is presented in Fig. 2. Observations were made more frequently than is practical in a service laboratory where tests are usually examined 3 to 4 hours after inoculation. Ninety-eight per cent (188 of 191) of those isolates which achieved coagulation did so within 4 hours after inoculation. However, these results affirm the value of keeping overnight for final reading those tubes negative after 4 hour incubation, a practice which has been recommended.[11, 12] As Fisher's[13] experience

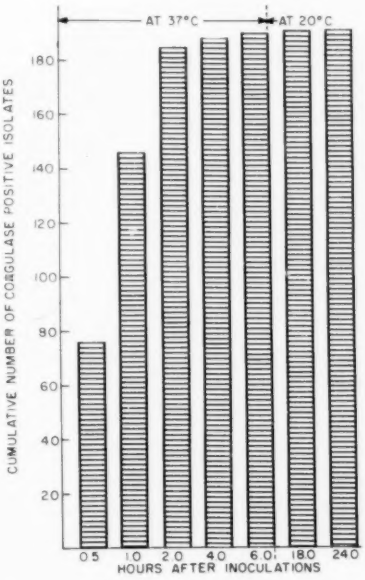

Fig. 2.—By 4 hours after inoculation, clotting had been achieved by 188 of the 191 clinical specimen isolates of staphylococcus which were coagulase-positive at 18 to 24 hours. No lysis of these clots occurred during the 24 hour period of observation.

TABLE I. THE REACTIONS OF CLINICAL SPECIMEN ISOLATES AND SKIN ISOLATES OF STAPHYLOCOCCUS

PARAMETERS		CLINICAL SPECIMEN ISOLATES: PATHOGENS (32 FROM PUS)	CLINICAL SPECIMEN ISOLATES: NONPATHOGENS (54 FROM UPPER RESPIRATORY TRACT)	SKIN STAPHYLOCOCCI (73 FROM ANTECUBITAL FOSSAE OF NORMAL SUBJECTS)
Tellurite	+	94%	87%	8%
	–	6%	13%	92%
Coagulase	+	91%	85%	7%
	–	9%	15%	93%
Mannitol	+	94%	91%	31%
	–	6%	9%	69%
Hemolysis	+	97%	91%	29%
	–	3%	9%	21%
Pigment	+	91%	83%	8%
	–	9%	17%	92%
Gelatinase	+	53%	43%	7%
	–	47%	57%	93%

Tellurite reduction, plasma coagulation, and pigment production, assayed as described in the text, reliably distinguished staphylococci obtained by culture of purulent lesions and upper respiratory tract from those staphylococci resident on the skin of normal individuals.

TABLE II. VIRULENCE TEST IN RABBITS BY INTRADERMAL INJECTION OF 11 ISOLATES OF STAPHYLOCOCCUS—THE SOURCE AND CHARACTERISTICS OF EACH ISOLATE

ISOLATE	SOURCE	*MINIMAL INTRADERMAL INFECTING DOSE	LABORATORY CHARACTERISTICS: TELLURITE	COAGULASE	MANNITOL	HEMOLYSIS	PIGMENT	GELATINASE
6	Skin	2.85×10^{10}†	–	–	–	–	–	–
13	Skin	3.50×10^{10}†	–	–	–	–	–	–
9	Skin	2.95×10^{10}†	–	–	+	–	–	–
16	Skin	2.20×10^{10}†	–	–	–	+	–	–
18	Skin	3.80×10^{10}†	–	–	+	+	–	–
32	Skin	3.75×10^{9}	–	–	–	+	+	–
28	Skin	2.00×10^{9}	+	+	+	+	+	–
241	Nose	2.50×10^{9}	+	+	+	+	+	+
245	Ear	2.65×10^{10}	–	–	+	–	–	+
242	Sputum	1.75×10^{10}	+	+	+	+	+	+
240	Pus	2.90×10^{9}	+	+	+	+	+	+

*Minimal intradermal infecting dose = smallest number of intradermally injected staphylococci which caused a lesion progressive to discharge of pus within 14 days after injection.

†Pustulation did not occur with these isolates in any dose given. The maximal doses employed are listed—these produced nodules about 2 mm. in diameter which were devoid of inflammation and were still palpable 14 days after injection. Resolution, without breakdown, was apparent in all (though not complete in every case) by 21 days after injection.

TABLE III. REACTIONS TESTED IN 15 BLOOD CULTURE ISOLATES

PARAMETER	ON CLINICAL GROUNDS: 7 PATHOGENS	8 NONPATHOGENS
Tellurite +	6	0
Coagulase +	6	0
Mannitol +	7	0
Hemolysis +	7	7
Pigment +	6	2
Gelatinase +	3	4

would predict, lysis of clots was not encountered in the 24 hour period of observation. Only a few of the antecubital skin staphylococci were capable of plasma coagulation (7 of 73). A detailed comparison of tellurite reduction and plasma coagulation, as accomplished by the 251 clinical specimen isolates of staphylococcus, is presented in Fig. 3. There is close agreement between these tests.

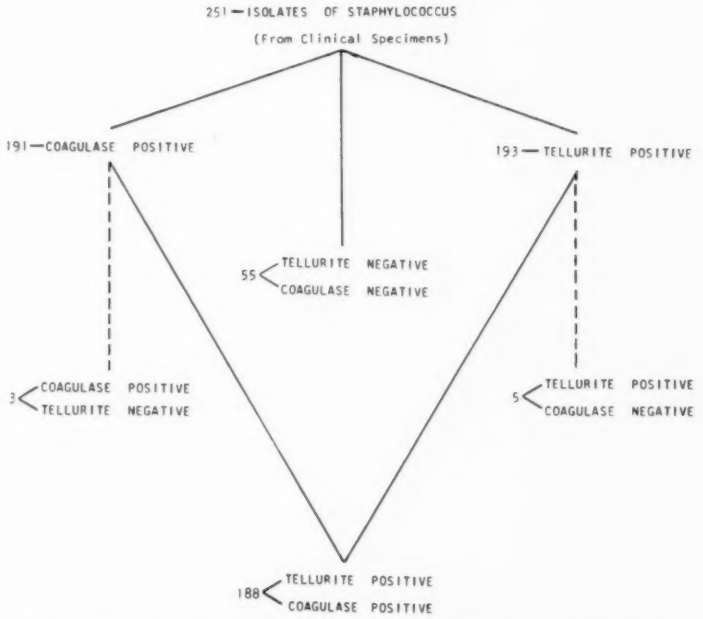

Fig. 3.—A detailed comparison of the ability of 251 clinical specimen isolates of staphylococcus to accomplish reduction of tellurite and coagulation of plasma indicates there is very close agreement between these tests.

DISCUSSION

First noted by Loeb[14] in 1903, the ability of certain staphylococci to coagulate the plasma of many animal species,[11, 15, 16] seemingly without regard to the anticoagulant used,[11, 15] has come to be regarded as a nearly uniform property of virulent staphylococci.[10, 11, 15-18] There are circumstances when avirulent staphylococci may be pathogenic, but these are unusual occurrences; for example, when they colonize damaged heart valves. Rabbit plasma is preferred for the coagulase test since, of readily available plasmas, it is the most susceptible to clotting by coagulase-positive staphylococci. With the demonstration that after storage as a dry, lyophilized powder, reconstituted plasma is a reliable reagent,[19] the coagulase test came within the capabilities of any laboratory. However, the preparation of reagents and test cultures, along with examination of tubes for clotting, still demands significant amounts of time. In addition, commercial dehydrated plasma costs about $0.09 per test.[20]

The data presented indicate that testing for tellurite reduction provides information of the same significance as testing for coagulase production. Moreover, ability to reduce tellurite is quite simply assayed. Isolates to be tested

are streaked on a sector of a tellurite-glycine agar plate; 8 tests are conveniently done on 1 plate. After overnight incubation at 37° C., the plates are inspected for the presence of jet black colonies indicative of growth with reduction of tellurite. With periods of incubation much in excess of 18 hours, some staphylococcal isolates which show no growth, or at most a grayish to brownish smudge in 18 hours, will develop black colonies. Not only is tellurite reduction conveniently carried out, but the cost per test is quite small—about $0.005 per test when 8 isolates are inoculated per plate.[21]

The presence in the tellurite-glycine agar medium of 1 per cent glycine, 0.5 per cent lithium chloride, as well as 0.02 per cent potassium tellurite makes for complete inhibition of gram-negative bacteria. Indeed, rather large inocula of staphylococcus are required for growth. As is shown in Table IV, 4 strains of staphylococci which were tellurite-positive by the sector streak method of examination were completely inhibited when inocula varying from 900 to 5,400 viable units were applied to the tellurite-glycine agar medium. These inocula were spread evenly over the entire surface area of 100 mm. in diameter Petri plate cultures, which were then incubated at 37° C. for 18 hours. In addition, our experience with swab cultures of the anterior nares and with stool specimens has indicated that the tellurite-glycine agar medium of Zebovitz and associates is not satisfactory for primary isolation of staphylococcus, since inoculation of duplicate specimens on a medium which included phenylethyl alcohol as a selective inhibitor consistently resulted in many more isolations of staphylococcus.

TABLE IV. INHIBITION OF STAPHYLOCOCCI, TELLURITE-POSITIVE BY THE SECTOR STREAK METHOD

ISOLATES	INOCULUM* IN VIABLE UNITS	AFTER 18 HOURS AT 37° C. ON TELLURITE-GLYCINE AGAR†
241	900	No growth
242	1,800	No growth
240	3,300	No growth
28	5,400	No growth

*Determined by pour plate colony count.
†Inoculum, in 1.0 ml. normal saline, was applied evenly to surface of medium.

Variation in the inhibitory potency of various batches of potassium tellurite has been reported.[5, 22] We have not encountered any difficulties on this score. It may be that the experience of Mueller and Miller[2] with *C. diphtheriae* inhibition by potassium tellurite has significance with regard to staphylococcus.

According to these authors, with a given concentration of potassium tellurite, the degree of inhibition of *C. diphtheriae* varied directly with the concentration of cystine in the medium. By using an acid hydrolysate of casein in their medium, Mueller and Miller obtained a replicable concentration of cystine, and the degree of tellurite inhibition was fixed by employing 0.01 per cent potassium tellurite. Similarly, in the tellurite-glycine agar medium, use of a pancreatic digest of casein as the nitrogen source fixes the cystine content of the medium, and, thus, protects against variation in the degree of staphylococcal inhibition by 0.02 per cent potassium tellurite.

Pigment production, in this study, appeared to be as good an indicator of whether a particular isolate was or was not potentially pathogenic as was either tellurite reduction or plasma coagulation. However, it should be noted that any off-white color in the colonies as examined by inspection was accepted as evidence of pigment production. Also, the conditions for development of pigment were not those readily adaptable to a hospital laboratory. The Chapman-Stone medium was, in our hands, a rather capricious formula. As we compounded the medium, almost every other batch was transparent and had to be discarded. The 48 hour incubation period required for full development of pigment on this medium imposes too great a delay in terms of time to permit practical use in a clinical laboratory. Pigment elaboration under the usual laboratory conditions (time and temperature of incubation, medium) is a property susceptible to variation.[23]

Hemolysis has been shown to vary with the species[19, 24] from which the erythrocytes were obtained. Arranged in decreasing order of fragility, they are rabbit, sheep, human, and horse. The concentration of erythrocytes in the medium is also quite important as are conditions of culture. At least 3 distinct hemolysins are elaborated by staphylococci; in the usual clinical laboratory, and under the conditions described in this study, α-hemolysin is most likely to be the enzyme responsible for the destruction of the red cells as observed. α-Hemolysin has not been proved to be of significance in the pathogenesis of staphylococcal infection, yet nearly all potentially pathogenic staphylococci do elaborate this enzyme. Some skin staphylococci were also hemolytic (Table II). It is possible that a more detailed analysis of hemolysin production, perhaps with quantitation of each hemolysin elaborated by each isolate, would permit better differentiation between pathogen and nonpathogen than was provided by simple testing for α-hemolysin. However, such detailed characterization is beyond the scope of a clinical laboratory.

Mannitol fermentation and elaboration of protease capable of hydrolyzing gelatin are enzymic feats more difficult to evaluate. The information adduced is of little value in differentiating potentially pathogenic staphylococci from nonpathogenic staphylococci (Table I).

The mechanism, by which reduction of tellurite is accomplished, is not known. When cell free filtrates prepared from broth cultures of known tellurite-positive staphylococci are inoculated on tellurite-glycine agar which is then incubated at 37° C., no tellurite reduction occurs. By microscopic examination the individual cocci making up black colonies on tellurite-glycine agar are black.

SUMMARY

1. Tellurite reduction, as determined by using a tellurite-glycine agar medium, is recommended for laboratory determination of potential pathogenicity of staphylococcus species isolated from clinical sources.

2. Tellurite reduction is of the same significance with regard to determination of potential pathogenicity as plasma coagulation (tube coagulase test).

3. The tellurite-glycine agar plate test offers the additional advantage of economy of time and materials.

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NEGATIVE CHRONOTROPISM AND INOTROPISM OF PROCAINE AMIDE IN THE HEART

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PROCAINE AMIDE is used clinically in the treatment of a wide variety of cardiac arrhythmias. The pharmacologic actions of procaine amide are qualitatively similar to those of the parent compound procaine. Procaine amide differs chemically from procaine in that the ester linkage is replaced by the amide grouping. Procaine amide is not as readily hydrolyzed by plasma esterases and possesses a longer duration of action than procaine. Fifty to sixty per cent is excreted unchanged in the urine; 2 to 10 per cent is excreted as free and conjugated para-aminobenzoic acid.[1] Information concerning the effect of procaine amide on the contractility of the myocardium is meager. McClendon and associates,[2] using the technique of cardiac catheterization, reported that the intravenous administration of procaine amide to human patients produced a fall in cardiac output, a fall in mean systemic arterial pressure, and a prolongation of the circulation time. On the basis of their indirect evidence they conclude that it exerts its major effect through a primary myocardial depressant action.

Marchetti and Dozio[3] added procaine amide in amounts of 100 to 400 mg. to the circulating blood of a Starling heart-lung preparation and were able to detect no change in the ability of a "well compensated" preparation to handle an increased venous inflow; however, if "failure" had been produced by prior administration of 100 to 200 mg. of pentobarbital sodium, then procaine amide further augmented the rise in venous pressure consequent upon increasing venous inflow. The present study was undertaken to determine whether procaine amide would have a negative inotropic effect in the heart-lung preparation under conditions in which the work load was constant.

This investigation was divided into 2 parts. The first series of experiments was concerned with establishing what effects procaine amide has on diastolic heart size and on cardiac rate under conditions of constant inflow-load and resistance-load in the dog heart-lung preparation. The second series of experiments was designed to determine whether procaine amide had a negative inotropic effect under conditions of constant inflow-load and resistance-load when a constant cardiac rate was maintained by electrical stimulation.

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METHODS

The dogs used weighed from 6 to 9 kilograms, and after light intravenous pentobarbital sodium anesthesia Starling heart-lung preparations[4] were set up by the usual methods. Eight preparations were studied. Changes in ventricular volume were recorded by use of a Henderson cardiometer and a piston-type mechanical volume recorder. Hearts of the animals used in the second series of experiments were driven at a constant rate by a square-wave stimulator (American Electronic Company) with a stigmatic salt-bridge string electrode placed upon the right atrium.

Determination of procaine amide concentration in plasma was done using the Kirsch and Strauss[5, 6] method for procaine and para-aminobenzoic acid. The method is based upon colorimetric determination of the colored compound resulting from diazotization of the para-amino group. In most cases the procaine amide was injected directly into the venous inflow in the right side of the heart. Fifteen minutes after addition of procaine amide to the circulation of the heart-lung preparation, blood was withdrawn in order to determine what concentration of procaine amide had been achieved.

RESULTS

The data from the first series of experiments are presented in Table I. There was a significant decrease in heart rate of all dogs after administration of procaine amide. Accompanying this decrease in heart rate was an increase in diastolic volume averaging 2.4 ml. per animal. Since, under the conditions of this experiment, there was a constant inflow-load, the question arises whether the increase in diastolic time did not permit greater venous filling, which could account for the increase in diastolic volume. This could obscure any direct negative inotropic effect of procaine amide upon the ventricular myocardium.

The data from the second series of experiments (Table II) demonstrate that negative inotropism is produced independently of changes in heart rate. In these experiments, with the heart rate remaining constant, an average increase in diastolic volume of 7.6 ml. was observed. It must, therefore, be concluded that procaine amide not only reduces heart rate, but also produces a negative inotropic effect by direct action on the ventricular myocardium.

TABLE I. EFFECT OF PROCAINE AMIDE ON CARDIAC RATE AND VOLUME IN THE HEART-LUNG PREPARATION WITH A CONSTANT INFLOW-LOAD AND RESISTANCE-LOAD

DOG	HEART RATE PER MINUTE: CONTROL	HEART RATE PER MINUTE: AFTER PROCAINE AMIDE	AMOUNT OF PROCAINE AMIDE GIVEN (MG.)	INCREASE IN DIASTOLIC VOLUME (ML.)	PLASMA LEVEL OF PROCAINE AMIDE ACHIEVED (MG. %)
1	118	102	100	1.5	9.0
2	85	73	50	2.5	3.7
3	111	102	50	2.0	2.6
4	111	94	40	2.5	2.5
5	153	118	60	3.5	4.5

TABLE II. EFFECT OF PROCAINE AMIDE ON HEART SIZE UNDER CONDITIONS OF CONSTANT RATE, CONSTANT INFLOW-LOAD, AND RESISTANCE-LOAD

DOG	HEART RATE PER MINUTE	AMOUNT OF PROCAINE AMIDE GIVEN (MG.)	INCREASE IN DIASTOLIC VOLUME (ML.)	PLASMA LEVELS OF PROCAINE AMIDE ACHIEVED (MG. %)
1	128	100	5.5	9.6
2	132	100	7.5	10.5
3	120	100	10.0	11.7

DISCUSSION

In this study there was no attempt to correlate the absolute increase in diastolic volume between the two series of animals. Larger amounts of procaine amide were used, and, consequently, higher blood levels were achieved in the second group of experiments. In general, the increase in diastolic size was greater in those animals with the higher plasma level of the drug (Table II), although the data are too few to attempt to establish a dose-response relationship. One striking exception to the above generalization occurred in Dog 1 (Table I). Although 100 mg. of procaine amide was administered, an increase in diastolic volume of only 1.5 ml. was observed. In this experiment the drug was added to the venous reservoir and thus the initial concentration of drug reaching the myocardium was not as high as that in the other experiments.

The question arises of how the levels of procaine amide achieved in these experiments compares with the levels reached in its clinical use. The lowest levels in Series I (Table I) which produced definite cardiac slowing and dilatation approximate the highest levels reached by Mark and associates,[1] which followed the intravenous administration of 1 Gm. of the compound. On the other hand the levels of procaine amide found in Series II (Table II) are about 8 times those reached when it is used in the usual way to convert an arrhythmia.[7]

Control studies demonstrated that the small amounts of sodium bisulfite and benzyl alcohol present in procaine amide solutions prepared for intravenous use in man do not have a negative inotropic effect in the dog heart-lung preparation.

CONCLUSION

In the dog heart-lung preparation with constant inflow-loading and resistance-loading, procaine amide produces both negative chronotropic and inotropic effects.

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MODIFIED RESIN METHOD FOR DETERMINATION OF RADIOIODINE CONVERSION RATIO

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THE radioiodine conversion ratio[1] has not proved itself to be as useful a test for the evaluation of thyroid function with radioiodine as was hoped originally. The disappointing results may be due, in part, to technical inadequacies in the performance of this test. In this report, a new method for determination of the conversion ratio is presented which is simple to perform and overcomes the disadvantages of prior methods.

The conversion ratio is the ratio of protein-bound I^{131} to total I^{131} in a sample of plasma usually obtained 24 hours after the administration of a tracer dose of sodium radioiodide. In this and similar tests, the protein-bound iodine in plasma usually is precipitated with trichloracetic acid. Zieve and Schultz[2] have shown that appreciable errors in the trichloracetic acid precipitation may occur as a result of loss of organic iodine when the precipitate is washed. Approximately 3 per cent protein-bound iodine is lost with each wash. Furthermore, inorganic iodide is trapped in the gelatinous precipitate. Zieve and Schultz[2] and others[3, 4] have proposed methods which utilize ion exchange resins to remove the inorganic iodide from plasma. In these methods, course, porous beads of resin are used which present a large surface to small molecules but a small surface to proteins. The beads also permit rapid flow rates which tend to reduce the absorption of protein on the resin. The moisture content of the resin columns must be held within narrow limits to prevent trapping of plasma in the resin or dilution of plasma with resin-held water.

We have found that even porous beads of resin bind a significant amount of protein while large amounts of inorganic iodide are not absorbed by the resin because of channeling and rapid flow. A modified resin method for determination of the radioiodine conversion ratio has been developed utilizing a nonionic detergent which prevents the binding of protein by ion exchange resins.[5] The method is applicable to other tests which require separation of protein-bound iodine from inorganic iodide.

METHODS AND MATERIALS

Modified Resin Method.—Approximately 1.5 g Dowex 1-X8, 50 to 100 mesh resin in the chloride cycle is suspended in distilled water, washed into a 8 mm. diameter polyethylene chromatograph tube (open at both ends), and allowed to drain. A number of tubes may be prepared simultaneously, stoppered, and stored at room temperature for future use.

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One milliliter of a solution of 3.0 per cent (w/v) Triton X-100* and 0.25 per cent (w/v) Hyamine 2389* in 0.035 M aqueous lithium chloride is added to 2 ml. plasma obtained from a patient, 24 hours after administration of a tracer dose of 60 μc sodium radioiodide. The radioactivity of the plasma mixture is measured. The plasma mixture is then run through the resin column completely. The tube in which the radioactivity of the plasma mixture was measured and the column are washed with 0.5 ml. of a solution of 1.0 per cent Triton X-100 in water, and the residual liquid in the column expressed by gentle, positive air pressure. The radioactivity of the resin-treated plasma is measured and, if necessary, a correction is made for the change in volume. This correction will depend on the geometry of the counting equipment used. The radioiodine conversion ratio is the ratio between the radioactivity of the eluate and the radioactivity of the plasma sample.

Other Methods.—The radioiodine conversion ratio was determined by the established resin method using Rezikits.†

Trichloracetic acid precipitation was also performed as follows: to 2 ml. plasma an equal volume of 20 per cent trichloracetic acid was added; the mixture was agitated with a glass stirring rod; the precipitate was centrifuged; and the supernatant was discarded. The precipitate was broken up and triturated vigorously with 3 ml. 10 per cent trichloracetic acid. A single washing was used to minimize loss of protein-bound iodine. Little inorganic iodide is removed by the second and subsequent washes.[2] The precipitate was dissolved in sufficient 1 N sodium hydroxide to reconstitute the original volume.

In Vitro Studies.—Sodium radioiodide, radioiodinated human serum albumin,‡ or radiothyroxine‡ was added to plasma obtained from four apparently healthy laboratory workers, and the conversion ratio of each of the twelve samples was determined by the three methods. Radiopure thyroxine was prepared by ascending chromatography on Whatman No. 1 paper using n-butanol saturated with 2 N ammonium hydroxide as developer.[6] The spot of radioactivity corresponding to thyroxine, $R_f = 0.58$, was cut out and the thyroxine eluted in 0.2 N sodium acetate and used immediately.

Studies in Patients.—In 58 patients seen in the Isotope Clinic for evaluation of thyroid function, the 24-hour thyroid uptake of radioiodine and radioiodine conversion ratio were determined by the trichloracetic acid precipitation method, by the usual resin method, and by the modified resin method. A determination of the status of thyroid function was made after evaluation of the history, physical examination, and other laboratory tests, including the radioiodine uptake, and was compared with the conversion ratio.

RESULTS AND DISCUSSION

The mean apparent conversion ratios measured by trichloracetic acid precipitation, by the usual resin method, and by the modified resin method after addition in vitro of sodium radioiodide, radioiodinated serum albumin, or radiothyroxine to plasma are presented in Table I. Small amounts of thyroxine added

TABLE I. APPARENT CONVERSION RATIO AFTER ADDING SODIUM RADIOIODIDE, RADIOIODINATED ALBUMIN, OR THYROXINE WAS ADDED TO PLASMA

RADIOACTIVE MATERIAL ADDED TO PLASMA	CONVERSION RATIO		
	TRICHLORACETIC ACID METHOD	USUAL RESIN METHOD	MODIFIED RESIN METHOD
Sodium iodide	7.2% (4.1 to 10.6)	8.6% (5.3 to 11.1)	2.3% (0.9 to 3.8)
Serum albumin	90.8% (88.6 to 93.2)	59.8% (51.6 to 71.8)	96.6% (94.4 to 99.0)
Thyroxine	92.4% (88.6 to 94.2)	71.8% (59.2 to 77.4)	93.3% (90.1 to 96.4)

The mean and range of four determinations are given.

*Obtained from Rohm and Haas, West Washington Square, Philadelphia, Pa.

†Dr. Paul Numerof, Squibb Institute for Medical Research, provided the Rezikits and polyethylene tubes used in this study.

‡From Abbott Laboratories, North Chicago, Ill.

TABLE II. THE 24-HOUR THYROID UPTAKE AND PLASMA CONVERSION RATIO IN PATIENTS

CLINICAL DIAGNOSIS	NUMBER OF PATIENTS	24-HOUR THYROID UPTAKE	CONVERSION RATIO		
			TRICHLORACETIC ACID METHOD	USUAL RESIN METHOD	MODIFIED RESIN METHOD
Hypothyroid	10	7.6 ± 5.0%	7.0 ± 3.2%	1.7 ± 1.1%	4.9 ± 2.6%
Euthyroid	33	28.2 ± 10.1%	18.8 ± 10.0%	8.4 ± 2.5%	20.2 ± 9.6%
Hyperthyroid	15	73.5 ± 13.7%	63.7 ± 32.6%	32.6 ± 17.8%	66.6 ± 15.3%

The mean and standard deviation of the mean are given.

to serum are almost entirely bound to protein.[7] Theoretically, the addition of sodium radioiodide should result in a zero conversion ratio, while addition of radioiodinated albumin or radiothyroxine should result in a conversion ratio of 100 per cent. Less inorganic iodide appeared in the protein-bound fraction with the modified resin method than with trichloracetic acid precipitation, or the usual resin method. In the usual resin method, a considerable amount of protein or thyroxine bound to protein is absorbed by the resin which leads to erroneously low values for the conversion ratio. With the modified resin method, passing the plasma mixture through more than one resin column resulted in a 2 to 3 per cent further loss of labeled albumin or labeled thyroxine, in contrast to the usual resin method where the apparent conversion ratio decreased by about the same fraction on each successive passage.

These observations are substantiated by our experiences in patients, which are summarized in Table II. Conversion ratios obtained by trichoracetic acid precipitation and by the modified resin method were comparable, in general. The modified resin method gave a better separation between the hypothyroid and euthyroid groups than the trichoracetic acid precipitation method. In the hypothyroid, the inorganic iodide trapped in the protein precipitate is comparable in amount to the protein bound radioiodine. Less trapping of inorganic iodide by the modified resin method may account for the better separation of hypothyroid and euthyroid groups. The usual resin method yielded significantly lower results than either of the other methods ($P < 0.01$, "t" test for differences between usual resin and other methods for both euthyroid and hyperthyroid groups); the greatest overlap between groups of different thyroid functional status occurred with the usual resin method.

The conversion ratios determined by the modified resin method and the 24-hour iodine uptake in 10 hypothyroid patients, 33 euthyroid patients, and 15 hyperthyroid patients are plotted in Fig. 1. When the results of these two tests are taken together, the patient groups are well separated with little overlap. The majority of hyperthyroid patients in this series had diffuse nodular enlargement of the thyroid gland (Plummer's disease); though abnormally high, the iodine uptakes and conversion ratios of such patients are lower than those of patients with Graves' disease. None of the patients in this series were known to have had kidney disease. In patients with advanced renal disease, factitiously low conversion ratios might be obtained as a result of delayed excretion of inorganic iodide.

The method of separating protein-bound substances from the free ions in plasma should be generally applicable and not limited to the specific example described in this paper.

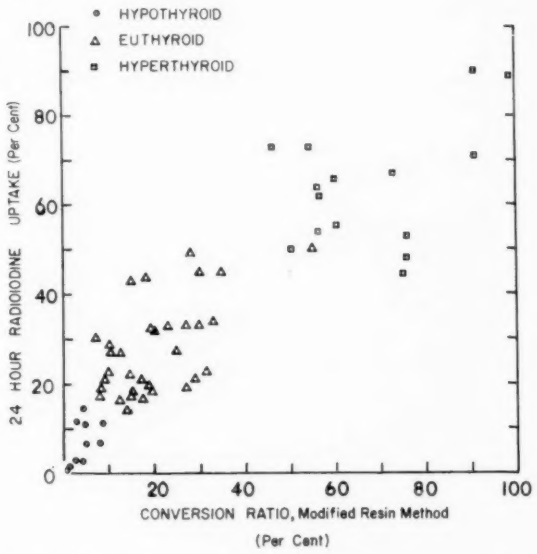

Fig. 1.—Relation of the 24-hour radioiodine uptake by the thyroid gland to the conversion ratio in hypothyroid, euthyroid, and hyperthyroid patients.

SUMMARY

A modified ion-exchange resin method for the determination of the radioiodine conversion ratio is described which utilizes a nonionic detergent to prevent the binding of protein by ion exchange resins. The method is simple to perform. Results in 58 patients are reported.

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LABORATORY METHODS

A PAPER SPOT TEST USEFUL IN STUDY OF HURLER'S SYNDROME

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DORFMAN[1] demonstrated the excretion of mucopolysaccharides, usually chondroitin sulfuric acid-B, by 17 of 18 children with Hurler's syndrome. He and his co-workers were able to isolate 150 to 200 mg. of acid mucopolysaccharide per 48 hours from urine of patients with this disorder, whereas only 20 to 50 mg. per 48 hours could be obtained from normal urine. For screening purposes a turbidimetric method in which acid mucopolysaccharides react with albumin at pH 3.0 was used. Meyer and associates,[2] using a method involving precipitation of acid mucopolysaccharides with cetyl trimethylammonium bromide, isolated a mixture of mucopolysaccharides from the urine of 4 out of 5 patients with gargoylism. Chondroitin sulfuric acid-B was the major component.

Chondroitin sulfuric acid can be detected on paper chromatograms using toluidine blue.[3, 4] Preliminary testing indicated that most normal urines contain no substance which reacts to give a positive test with toluidine blue. Separation of chondroitin sulfuric acid by chromatographic migration seemed unnecessary in a screening test for the presence of this substance in urine.

METHODS

To perform the test 5, 10, and 25 μl urine are placed on separate spots on a piece of Whatman No. 1 filter paper. A micropipette is used to add 5 μl at a time. Each application should dry thoroughly before the next is made. The paper is dipped in an aqueous solution of 0.04 per cent toluidine blue* buffered at pH 2.0 for about 1 minute, drained, and rinsed in 95 per cent ethyl alcohol.

Urine from children with Hurler's syndrome gave a purple spot against a light blue background. Known amounts of chondroitin sulfuric acid were applied to paper, as spots of the same diameter as the urine spots. Five microliters of a solution of chondroitin sulfuric acid containing 0.10 mg. per milliliter gives a positive test. A comparison of the amounts of chondroitin sulfate in urine with known amounts gives an estimate of the concentration.

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*Coleman certified buffer tablets may be used. The reagent should be stored in a refrigerator to prevent mold growth.

RESULTS

Chondroitin sulfuric acid concentration in urine of children with Hurler's syndrome is given in Table I.

Patients 6 and 7 were siblings. Patients 2 and 8 had mild forms of the disease. The specimen from patient 4 was a dilute urine (specific gravity 1.005). Creatinine ratios can be used to correct for variations in urine volumes when random specimens are tested. The volume of urine required to give a positive test from children with Hurler's syndrome contained between 2 and 5 mcg. creatinine.

Approximately 250 urine specimens from children hospitalized for a variety of diseases were tested using urine volumes ranging from 25 to 60 μl, depending on the volume required to contain 40 mcg. creatinine. When these larger volumes were used a faintly positive test was noted in urines from a diabetic child, a child with phenylketonuria, and a male sibling of Patient 4. Urine specimens from a newborn infant gave positive tests for chondroitin sulfate on the second, third, and fourth days of life, though not on the first. Urine specimens from this infant were negative by the third week. Urine from 2 female siblings of this infant also gave faintly positive tests when volumes of urine containing 40 mcg. creatinine were used. One of the sisters, age 4 years, has galactose disease for which she has been treated with a lactose-free diet since the age of 3 months. Urine from 5 other newborn infants gave positive tests during the first week of life. In each instance the concentration of chondroitin sulfate was estimated to be one-eighth to one-twentieth that found in urine of children with Hurler's syndrome.

The difference demonstrable with this test between chondroitin sulfate in urine of normal children and of those with Hurler's syndrome appears to be in the amount excreted. If a small volume of urine is tested, 5 μl, or that volume containing approximately 5 mcg. creatinine, the amount of chondroitin sulfate present in urine of normal children older than 1 week would not be sufficient to give a positive test under the conditions described.

Urine specimens dried on filter paper had been obtained from 2,200 infants between the ages of 2 weeks and 6 months as part of a urine survey for detection of phenylketonuria.[5] These furnished a source of urine to test for excretion of chondroitin sulfate by young infants. As a control, filter paper was dipped in urines that gave positive tests for chondroitin sulfate when a volume of 5 μl was used. From these dried urine papers, pieces approximately ½ inch square

TABLE I. EXCRETION OF CHONDROITIN SULFURIC ACID BY CHILDREN WITH HURLER'S SYNDROME

CASE NO.	SEX	MG. PER ML.
1	F	0.5
2	M	0.5
3	M	0.2
4	M	0.1
5	M	0.2
6	M	0.5
7	F	0.5
8	M	0.2
9	M	0.75

were cut, dipped in reagent, and washed. The urines from children with Hurler's syndrome again gave positive tests. The control papers were kept at room temperature and continued to give a positive test for at least a month. Small sections were cut from each of the 2,200 urine filter paper specimens and tested. Four positive tests were obtained. Of the children, all under 3 months of age when first specimens were obtained, none appeared to have gargoylism on examination. Six months later 3 of the children have no abnormalities. One of the children was operated upon for excision of an encephalocele. Urine specimens from this child following the operation were still positive for chondroitin sulfuric acid. The child continued to have convulsive seizures 6 months later.

DISCUSSION

The metachromatic effect observed with toluidine blue is due to a direct combination of acidic functional groups on the polysaccharide polymer with basic groups of the dye. The reagent which is described is buffered to prevent staining of compounds which are ionized above pH 2. Nevertheless, many substances other than chondroitin sulfuric acid may produce metachromasia. Separation and identification of the substances reacting with toluidine blue will be necessary to clarify the role of acid mucopolysaccharide excretion in gargoylism. Excretion of chondroitin sulfate by children with Hurler's syndrome may not be a characteristic feature of the disease, nor is it necessarily limited to those children with the disease. The excretion of mucopolysaccharides in any considerable amount beyond the newborn period is certainly not common. In our sampling, positive tests were obtained in 0.2 per cent of the urine specimens tested. If one recognizes the limitations of this simple spot test as a screening method it should be of value as an additional diagnostic tool in the study of gargoylism.

SUMMARY

A paper spot test using toluidine blue reagent is described for the detection of chondroitin sulfuric acid. Urine specimens from 9 patients with Hurler's syndrome gave positive tests. Two hundred and fifty urine specimens from children with a variety of diseases were tested using urine volumes 5 to 10 times that which gave positive tests in cases of Hurler's syndrome. Faintly positive tests were obtained in 4 specimens. From a sampling of 2,200 urine specimens from infants between the ages of 2 weeks and 6 months, 0.2 per cent positive specimens were obtained. Urine from newborn infants during the first few days of life gave positive tests.

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AN EVALUATION OF SEVERAL MEDIA FOR THE EARLY DETECTION OF PSEUDOMONAS AERUGINOSA ENCOUNTERED IN CLINICAL PRACTICE

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THE increasing clinical importance of the pseudomonads in both acute and chronic infection has been reported in recent years.[1, 2] The only known human pathogen among this genus of bacteria is the type species, *Pseudomonas aeruginosa*. The in vivo emergence of strains of this organism which are resistant to many of the common antibiotics used in therapy is probably the primary reason for the Pseudomonas problem. In this fashion, it resembles the Staphylococcus problem.

One of the principal characteristics of *Pseudomonas aeruginosa* is its soluble blue-green pigment. This pigmentation characteristic may be considered pathognomonic for this species when encountered in clinical specimens. Therefore, in clinical bacteriologic laboratory practice, this pigmentation criterion may be considered diagnostic for *Pseudomonas aeruginosa*. Other tests for the identification of this organism, besides being time consuming, become supplementary and merely confirmatory.

The chief difficulty in the rapid identification of this organism is the fact that many strains isolated from clinical material do not produce pigment on the isolation medium. Because of this, an extra day is required for the usual routine identification procedures. Three biochemical tests have been developed in recent years to aid in the identification of this organism. Two of these tests, the Haynes gluconate oxidation test, and the Gaby cytochrome oxidase test, require as much time for identification as do the usual routine methods. The third, Kovac's oxidase test, has been described by Gaby and Hadley[3] as being incapable of separating *Alkaligenes faecalis* from *Pseudomonas aeruginosa*, in several instances. These biochemical tests all require time for growth of the organism before they can be accomplished, in addition to the extra time and effort required to perform the actual test.

In our opinion, if the strain of *Pseudomonas aeruginosa* produces pigment on the isolation medium, visual observation of this characteristic provides the most rapid and reliable method for identifying this organism. For this reason,

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TABLE I. DISTRIBUTION OF PSEUDOMONAS AERUGINOSA STRAINS

ISOLATION SOURCES	ISOLATION MEDIA*	NUMBER OF STRAINS
Urine	BA(27), EMB (6)	33
Burn wounds	BA	20
Ear	BA	15
Feces	SS	8
Throat	BA	6
Sputum	BA	2
Lung	BA	2
Trachea	BA(1), SS (1)	2
Brain abscess	BA	1
Scalp	BA	1
Chin	BA	1
Blister	BA	1
Abdominal wound	BA	1
Vaginal cyst	BA	1
Eye (rat)	BA	1
ATCC	Unknown	7
Total		102

*BA = blood agar; EMB = eosin-methylene-blue agar; SS = salmonella-sheigella agar.

we decided, as a preliminary study, to compare the pigment-promoting ability of the usual media reported in the literature for the isolation of *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Several clinical bacteriologic laboratories in this geographic area were requested to submit cultures of *Pseudomonas aeruginosa* isolated from clinical materials.

The diagnostic procedures for isolation and identification of this bacterium varied in the several collecting laboratories. In general, 2 days, and usually more, elapsed before definite identification could be made. The distribution of the 102 strains collected is shown in Table I. The widespread occurrence and infectivity of this organism in the human body is apparent. It is noted that 7 strains of *Pseudomonas aeruginosa*, secured from the American Type Culture Collection, were included in this study. The medium used in the primary isolation of approximately 80 per cent of the total strains was blood agar.

Upon receipt of the Pseudomonas isolates at the School of Aviation Medicine, each specimen was purified by subculture on a nutrient agar streak plate incubated for 48 hours at 37° C. Growth from a single colony on each plate was transferred to each of 2 nutrient agar slants and the latter was incubated for 24 hours at 37° C. One set of 102 nutrient agar slants was sent to the Lackland Group (Laboratory B), the other set was retained by the School of Aviation Medicine Group (Laboratory A). Each group carried out an identical procedure. Each strain was streaked on each of the 3 media studied. Following 24 hours' incubation at 37° C. the 3 plates for each strain were visually observed and compared against one another with regard to pigmentation. An arbitrary system of 0 to 4 plus was set up in each laboratory to quantitate pigmentation.

Three media were studied. Nutrient agar was selected as representative of the routine media commonly used in diagnostic bacteriology. Asparagine agar was reported several years ago by Mitchell and others[4] as ideally suited for demonstrating the pigmentation of the Pseudomonas. Recently, Martineau and Forget[5] reported the use of Sabouraud maltose agar as an effective medium for Pseudomonas pigmentation.

RESULTS

A comparison of the 3 media for the diagnostic detection of pigmentation in 102 strains of *Pseudomonas aeruginosa* recorded by the 2 laboratories is shown in Fig. 1. Laboratory A recorded almost 95 per cent of the strains as

showing pigmentation when asparagine agar was used, whereas Laboratory B showed considerably different results. Both laboratories demonstrated the failure of nutrient agar to detect diagnostically the strains that show pigmentation. On the other hand, the effective ability of Sabouraud maltose agar to detect almost 95 per cent of the strains as pigmenters is apparent.

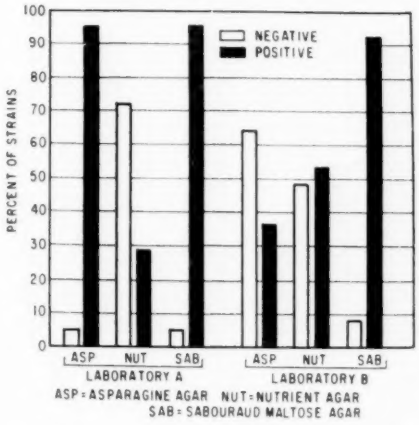

Fig. 1.—Comparison of media for the diagnostic detection of pigmentation of 102 strains of *Pseudomonas aeruginosa* after 24 hours.

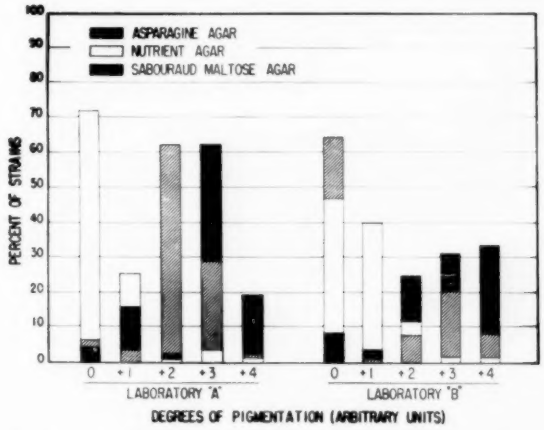

Fig. 2.—Comparison of pigmentation intensity produced by each of 102 strains of *Pseudomonas aeruginosa* on different media.

Fig. 2 shows a comparison of the degree of pigmentation exhibited by the 102 strains of Pseudomonas on the 3 different media as recorded by the 2 laboratories. The first point to be made is that the quantitation differed between the 2 laboratories. This is as might have been expected with such a subjective criterion as visual pigmentation. Nevertheless, it is apparent that both laboratories evaluated Sabouraud maltose agar as the medium showing the greatest intensity of pigment in the largest per cent of the strains examined. It is also

noted, as shown in Fig. 1, that the 2 laboratories differed appreciably on their analyses with asparagine agar. Further, the failure of nutrient agar to produce a high degree of pigmentation was noted in both laboratories.

TABLE II. CORRELATION BETWEEN LABORATORIES OF PIGMENTATION OBSERVED AFTER 24 HOURS FOR 102 STRAINS OF PSEUDOMONAS AERUGINOSA ON DIAGNOSTIC MEDIA

	PER CENT AGREEMENT			PER CENT DISAGREEMENT		
	PIGMENTATION			PIGMENTATION		
MEDIUM	NEGATIVE	POSITIVE	TOTAL	LAB. A POS. LAB. B NEG.	LAB. A NEG. LAB. B POS.	TOTAL
Asparagine agar	5.2	34.0	39.2	60.8	0.0	60.8
Nutrient agar	32.4	15.6	48.0	13.7	38.3	52.0
Sabouraud maltose agar	4.8	92.2	97.0	3.0	0.0	3.0

Table II shows the results of the 2 separate laboratories' investigations on each strain of *Pseudomonas* on each of the 3 media. The primary fact noted here is that the 2 laboratories agreed as to negative or positive pigmentation in 97 per cent of the strains that were examined when Sabouraud maltose agar was used. The results obtained with the other 2 media indicate a significant degree of difference in findings between the 2 laboratories. This would tend to limit the usefulness of these media for rapid diagnostic bacteriologic tests involving *Pseudomonas aeruginosa*.

DISCUSSION

The principal function of the clinical diagnostic bacteriologic laboratory is the completion of requested analyses. Such results are of greatest aid to the physician when they can be performed with a minimum of time and a maximum of accuracy. Therefore, early, reliable results from the bacteriologic laboratory will help the physician in making decisions as to treatment and control of infectious diseases.

It is believed that the study reported here provides a reasonable answer to one small, but important, problem in diagnostic bacteriology, namely, the pseudomonads. It is reasoned that any technique which will increase the chances for early detection and reporting of *Pseudomonas aeruginosa* warrants consideration by clinical bacteriologists. Further, since technicians in clinical laboratories, as a group, show an extremely wide variation in competency and experience, it is imperative that those tests which assure the greatest chances for accurate analyses be employed. It is suggested that the routine use of Sabouraud maltose agar for specimens from certain selected specialties of the medical and surgical services of the hospital may effectively aid in the diagnosis, treatment, and care of the patient.

SUMMARY

This report describes an evaluation of several media for the early detection of *Pseudomonas aeruginosa* that may be encountered in clinical specimens. The criteria chosen were qualitative and quantitative pigmentation. The deficiencies possessed by the usual laboratory media, as represented by nutrient agar, have been shown. The ability of Sabouraud maltose agar to increase the chances for

detecting pigmentation qualitatively, as well as increasing the degree of pigmentation of strains of *Pseudomonas aeruginosa*, is documented in these studies. It is suggested that careful consideration be given to this medium for its routine incorporation in diagnostic bacteriologic procedures in selected clinic specialties.

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THE CLINICAL APPLICATION OF GAS CHROMATOGRAPHY TO THE ANALYSIS OF RESPIRATORY GASES

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THERE is need for a rapid and reliable method for the qualitative and quantitative analyis of respiratory gases. The methods of Haldane as well as the micro technique devised by Scholander[1] are time consuming and require a trained analyst. The microanalysis techniques, though useful, are difficult when a high concentration of gas is undergoing absorption. The addition of anesthetic gases to the usual respiratory gases makes accurate determinations by any volumetric method difficult. Infrared analysis cannot analyze other components of the respiratory gas specimens.[2] For these reasons, the application of gas adsorption chromatography for clinical requirements was evaluated.

The principal application of differential adsorption for purposes of analysis dates back to 1897 when D. J. Day showed that crude petroleum when forced upward through a column of powdered limestone became fractionated, and the oil that first emerged was thin and lighter in color than that which followed. This was initially termed "adsorptive filtration."[3-5] Since that time, and particularly in the last ten years, this principle has been used extensively[6-8] in the separation of substances for analytical purposes. It has been limited mainly by lack of invention or improvement of means of detection. The use of one of these methods, gas adsorption chromatography, was used in the study to investigate concentrations of respiratory gases ordinarily encountered in clinical work.

MATERIALS AND METHODS

The Beckman gas chromatograph (Model No. 178) was evaluated (Fig. 1). This instrument allows the analysis of a gaseous mixture by injecting the gas which is undergoing analysis into a column that has a carrier gas circulating through it. The column has a packing for which components of the unknown gas have different affinities; this results in the components traveling at different rates through the column. They arrive separately at the column exit where they are measured by a suitable detector. Helium is used as the carrier gas in this particular instrument and enters the inlet at the upper left of the flow diagram (Fig. 2). The carrier gas flows through a pressure regulator and then through a special filter to a capillary orifice. The regulated flow then passes through one-half of the thermal conductivity detector cell where it serves as a reference. Gas

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samples are introduced through the sampling valve and are carried through the coiled adsorption column. As each component exits from the column, it passes through the second half of the thermal conductivity detector cell where it is compared with the reference. The difference in thermal conductivity of the 2 sides of the cell produces a voltage imbalance which is transmitted to a Bristol recording system (Model No. 560).

In this study the chromatograph attained a constant temperature of 35° C. by means of a thermostatically controlled heating element; the columns were flushed with the helium carrier gas. A standard 5 liter bag was filled with varied concentrations of oxygen and carbon dioxide. Using a three-way stopcock and an oiled syringe, samples were removed from the bag and 5 ml. was injected into the sampling chamber. After allowing 5 seconds

Fig. 1.

for the gas to equilibrate to atmospheric pressure, the sampling chamber allowed exactly 1.2 ml. of the sample to be added to the carrier gas in the column. The carrier gas flowed at 90 ml. per minute for oxygen analysis with a 4 foot Linde 13X (No. 17337) column, and at 48 ml. per minute with a 12 foot Di n-Butyl Maleate (No. 17329) column for carbon dioxide analysis. A constant current of 250 milliamperes was used in the detector cell. The peak height and time of elution were recorded. The same sample was then analyzed by the standard Scholander micro method. All samples were analyzed in quadruplicate with the chromatograph and in duplicate by the Scholander method.

RESULTS

Utilizing the described technique, the apparatus was calibrated for analysis of oxygen and carbon dioxide. Peak height of gas samples, as measured on the Bristol recorder, was plotted as the ordinate and per cent oxygen and

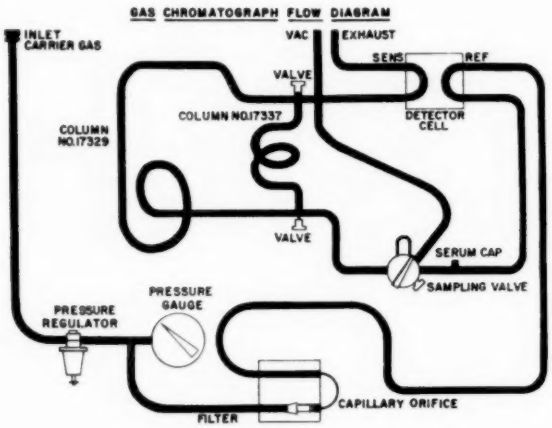

Fig. 2.

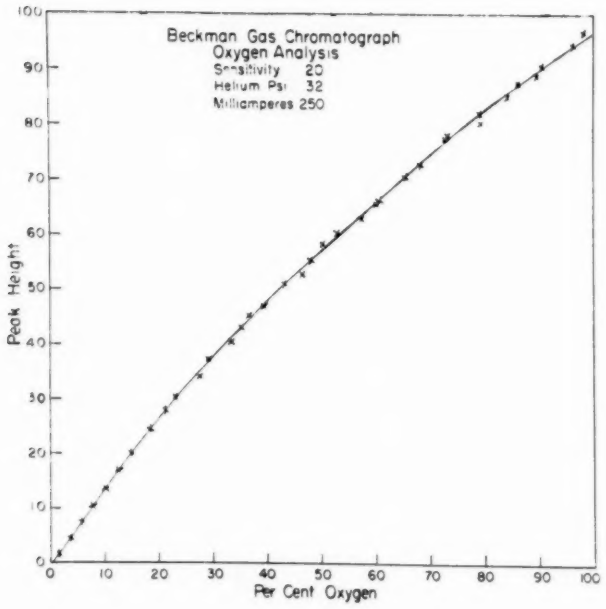

Fig. 3.

carbon dioxide as the abscissa (Figs. 3 and 4). Per cent oxygen was calibrated in the range 0 to 100 per cent and carbon dioxide in the range of 0 to 10 per cent. In these ranges an almost linear relationship exists between

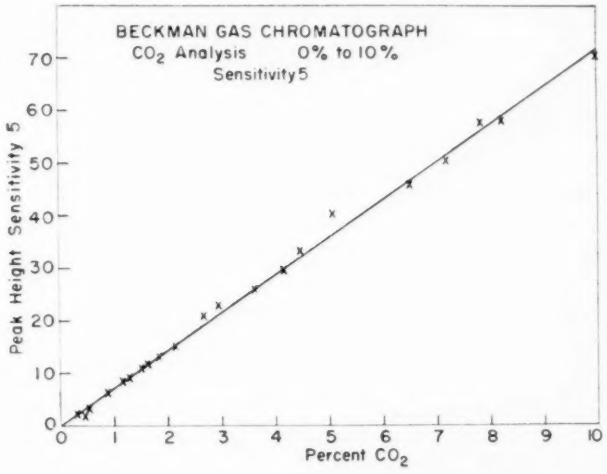
BECKMAN GAS CHROMATOGRAPH
CO_2 Analysis 0% to 10%
Sensitivity 5
Peak Height Sensitivity 5
Percent CO_2
0
10
20
30
40
50
60
70
0 1 2 3 4 5 6 7 8 9 10

Fig. 4.

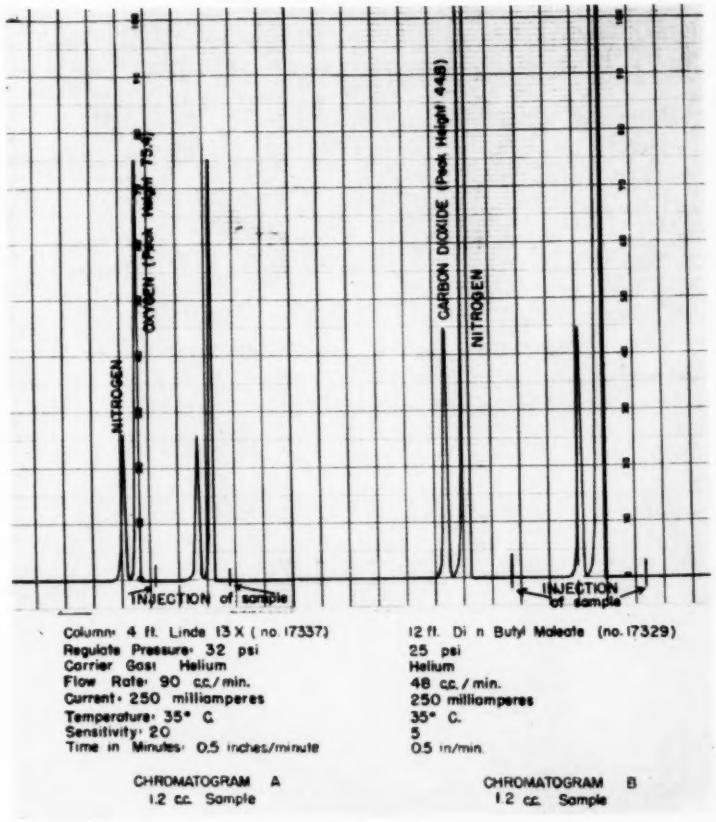
NITROGEN
OXYGEN (Peak Height 73.4)
CARBON DIOXIDE (Peak Height 44.8)
NITROGEN
INJECTION of sample
INJECTION of sample
Column: 4 ft. Linde 13 X (no 17337)
Regulate Pressure: 32 psi
Carrier Gas: Helium
Flow Rate: 90 cc./min.
Current: 250 milliamperes
Temperature: 35° C.
Sensitivity: 20
Time in Minutes: 0.5 inches/minute
12 ft. Di n Butyl Maleate (no. 17329)
25 psi
Helium
48 cc./min.
250 milliamperes
35° C.
5
0.5 in/min.
CHROMATOGRAM A
1.2 cc. Sample
CHROMATOGRAM B
1.2 cc. Sample

Fig. 5

peak height and per cent concentration. Typical recordings (Fig. 5) reveal peak heights as measured directly on the recorder paper and demonstrate the ease with which results are reproducible.

DISCUSSION

The gas adsorption chromatograph offers a rapid and reliable method for the qualitative and quantitative analysis of respiratory gases. The amount of sample required for analysis is small. For analysis of oxygen and carbon dioxide, 2 columns are utilized. The carrier gas as well as the gas undergoing analysis is easily directed into either column by means of valves. The simplicity of operation, compared to other standard methods of gas analysis, allows multiple determinations during experimental studies with a high degree of accuracy. Other components of ventilatory gases, including anesthetic gas, can be determined both quantitatively and qualitatively with the proper selection of column packing materials. The error in repeated analysis of carbon dioxide never exceeded 0.1 volume per cent and the maximum error in oxygen determinations was 0.8 volume per cent in the higher concentrations of the gas. All results were reproduced at various time intervals throughout the period of study and the determinations are available within 2½ minutes after injection into the chromatograph.

SUMMARY AND CONCLUSIONS

A method of qualitative and quantitative analysis of respiratory gases, oxygen and carbon dioxide, using the Beckman gas adsorption chromatograph (Model No. 178), has been described. Accuracy within the range of clinical application has been determined to be within 0.1 volume per cent for carbon dioxide and within 0.8 volume per cent for oxygen. Five milliliters of gas sample is sufficient for an accurate analysis. Results are available within 1 minute for oxygen, and within 2½ minutes for carbon dioxide.

The gas adsorption chromatograph provides a reliable and accurate apparatus for the rapid analysis of small quantities of respiratory gas.

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AN ABBREVIATED METHOD FOR BLOOD VOLUME MEASUREMENT EMPLOYING RADIOCHROMIUM

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THE determination of the intravascular volume has many clinical applications, but the existing methods are not completely satisfactory for routine use. Evans blue dye (T-1824) and radioactive iodinated human serum albumin (RISA) have been extensively employed, but the volume of distribution of these substances is considerably greater than the intravascular space, particularly under circumstances where the integrity of the vascular tree might be compromised. The method in which radiochromium-labeled red blood cells are used is much more accurate, but, because the labeling process is time consuming, this method has enjoyed only limited clinical application.

It was the purpose of the present study to modify the tagging procedure so that the method might be employed in emergency situations without seriously compromising its accuracy. The entire procedure has been shortened so that the material can be prepared for injection within 20 minutes, and accurate blood volume determinations can be provided in an additional 50 minutes or less.

MATERIALS AND METHODS

Rapid Method.—To 40 μc of $Na_2Cr^{51}O_4$ in 1 ml. saline in a sterile 30 ml. test tube or serum bottle was added 15 ml. whole blood, which had been collected in a syringe containing 3 ml. of a citrate-dextrose solution.* This was incubated at room temperature for 5 minutes, filtered with mild suction through any blood administration set, and washed once with 15 ml. saline. The cells were then reconstituted with saline to a volume of approximately 15 ml.

Ten milliliters of this tagged-cell suspension was injected intravenously using a calibrated syringe. Fifteen minutes after injection, a heparinized sample was collected for preparation of a 4 ml. whole blood sample and duplicate Wintrobe hematocrits.

The standard was prepared by adding 3 ml. of water† to 1 ml. of the reconstituted, tagged cells. If doses of 40 μc or less are employed dilution of the standard is unnecessary. Counting was done in a sodium iodide (thallium-activated), crystal scintillation well counter having a counting efficiency of 1,400 counts per second per microcurie Cr^{51}.‡

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*Baxter Laboratories, Inc., ACD solution (Transfuso Vac Bottle). Each 100 ml. contains: dextrose USP, 1.47 Gm.; sodium citrate USP, 1.32 Gm.; citric acid USP, 0.48 Gm.

†Water hemolyzes the cells and assures uniform distribution of the activity within the 4 ml. volume; the whole blood samples are thoroughly mixed before each counting.

‡The calculations are as follows:

$$\text{blood volume} = \frac{\text{count of standard} \times \text{milliliter injected}}{\text{count of sample} \div 4}$$

red cell mass = blood volume × hematocrit.
plasma volume = blood volume − red cell mass.

TABLE I. COMPARISON OF RESULTS USING A STANDARD AND RAPID METHOD

PATIENT	STANDARD METHOD				RAPID METHOD (EQUILIBRATION TIME 15 MIN.)			% DIFFERENCE			TIME ELAPSED BETWEEN DETERMINATIONS
	BLOOD VOLUME	RED CELL MASS	PLASMA VOLUME	EQUILIBRATION TIME	BLOOD VOLUME	RED CELL MASS	PLASMA VOLUME	BLOOD VOLUME	RED CELL MASS	PLASMA VOLUME	
1	4,710	2,470	2,240	30 seconds	4,720	2,380	2,340	+0.2	-3.6	+4.5	2 hours
2	3,560	1,620	1,940	1 minute	3,560	1,640	1,920	0.0	+1.2	-1.0	15 minutes
3	5,390	2,610	2,780	2 minutes	5,190	2,490	2,700	-3.7	-4.6	-2.9	2 hours
4	5,410	2,320	3,090	2 minutes	5,230	2,350	2,880	-3.3	+1.3	-6.8	3 hours
5	3,210	1,680	1,530	1 minute	2,960	1,580	1,380	-7.8	-6.0	-9.8	1 hour and 30 minutes
6	3,720	2,130	1,590	15 seconds	3,640	2,080	1,560	-2.2	-2.3	-1.9	15 minutes
7	6,520	2,940	3,580	15 seconds	6,280	2,890	3,390	-3.7	-1.7	-5.3	15 minutes
8	3,750	1,320	2,430	15 seconds	3,930	1,330	2,600	+4.8	+0.8	+7.0	15 minutes
9	3,910	2,260	1,650	15 seconds	3,760	2,180	1,580	-3.8	-3.5	-4.2	15 minutes
10	4,770	2,140	2,630	15 seconds	4,460	2,120	2,340	-6.5	-0.9	-11.0	15 minutes

TABLE II. THE EFFECT OF THE ANTICOAGULANT ON THE LABELING OF RED CELLS

PATIENT	HEMATOCRIT	% UPTAKE OF Cr^{51}, BY RED CELLS IN WHOLE BLOOD AFTER 5 MINUTES' INCUBATION		
		ABBOTT ACD	BAXTER ACD	HEPARIN
1	42.5	84.7	66.0	32.8
2	44.5	90.2	82.7	44.1
3	45.0	80.3	69.5	40.3
4	47.8	85.3	75.7	47.4

TABLE III. COMPARISON OF LABELING IN BAXTER ACD AT VARIOUS HEMATOCRIT LEVELS AND EFFECT OF MULTIPLE WASHINGS

HEMATOCRIT	% UPTAKE OF Cr^{51} BY RED CELLS IN WHOLE BLOOD AFTER 5 MINUTES' INCUBATION	
	% UPTAKE (3 WASHES)	% REMAINING WITH CELLS AFTER 1 WASH
10.0	23.3	24.0
30.0	64.8	66.3
50.0	84.9	86.4
70.0	91.4	93.2

Standard Method.[1]—The standard procedure departs from the above method as follows: heparin was the anticoagulant used; the plasma was removed before the addition of Cr^{51}; the incubation time was 30 minutes at 37° C.; the cells were washed twice, and a 45 ml. cell suspension was injected.

Effect of Anticoagulants on Cr^{51}-Labeling.—Fifteen milliliter whole blood samples were collected in 3 ml. Abbott ACD (special formula), 3 ml. Baxter ACD, and in 0.05 ml. heparin (10 mg. per milliliter), incubated with 40 μc Cr^{51} for 5 minutes at room temperature, and washed 3 times with cold saline.

Comparison of Labeling in Baxter ACD at Various Hematocrit Levels and Effect of Multiple Washings.—Eighty milliliters blood was collected in 17 ml. Baxter ACD, and, after adjusting to hematocrits of 10, 30, 50, and 70 per cent, 15 ml. aliquots were incubated with 40 μc Cr^{51} for 5 minutes at room temperature. The cells were counted after 1 and 3 saline washings.

RESULTS

Comparison of the 2 methods shows excellent agreement in values for plasma and red cell mass volumes, particularly the latter, which is the least susceptible to variation between determinations. The sequence of the 2 types of determinations was alternated, resulting in time intervals of from 15 minutes to 3 hours between determinations.

As can be seen in Table II, labeling efficiency of the Abbott ACD solution (special formula) is slightly superior to that of the Baxter formula. The latter was employed because it was readily available and more economical.

The data in Table III demonstrate 2 technical points: (1) repeated washings with saline provide negligible reduction in unlabeled Cr^{51} content compared with that accomplished by a single washing; and (2) the decreased per cent uptake observed in extremely anemic states may necessitate a correspondingly increased Cr^{51} dosage.

DISCUSSION

Various methods have been proposed for the measurement of blood volume using radioactive chromium. Among the modifications which deserve consideration is the use of labeled low titer Group O, Rh-negative cells[2]; however it is sometimes difficult to maintain a supply at all times for emergency use.

Economic considerations resulted in exclusion of the method of counting samples in a special plastic coil.[2, 4, *] Not only was washing the coil between samples rather time consuming, but the counting efficiency was reduced to 38 per cent of the efficiency of a 4 ml. sample. Such a decrease in count rate would of necessity either elevate the amount of $Na_2Cr^{51}O_4$ used in labeling or would prolong the time required for counting.

The injection of unwashed and unfiltered cells following the addition of ascorbic acid has also been proposed.[5] If such a method is adopted certain facts should be considered: (1) on occasion small fibrin clots are observed in the filtering sets; (2) it has been established that leukocytes[6] and platelets[7] are labeled with Cr^{51} and if either of these components is elevated, care should be taken to remove them while aspirating the saline wash. The

*Radicoil (Abbott Laboratories).

necessity of eliminating the leukocytes before injection became apparent when a 220 per cent error was made in a blood volume determination on a patient having a leukocyte count of 400,000 c.mm. (3) Hemolysis, although uncommon, may not be perceived unless centrifugation is employed.

The results of this study demonstrate that the procedure commonly employed to label red cells with Cr^{51} may be shortened without seriously compromising the accuracy of the method. The procedure, thus shortened, may be quickly performed under circumstances which are considered emergent, yet at the same time the accuracy of the original method may be retained substantially.

This method has been applied in pre- and postoperative blood and fluid replacement problems. The rapidity with which the blood volume may be determined by this method should widen its field of usefulness. The method accurately measures only the red cell mass; the plasma and total blood volumes are estimated from the venous hematocrit.

SUMMARY

The procedure for determining the blood volume in which radiochromium is used has been modified for the purpose of completing the determination in a minimum length of time without significantly compromising the accuracy of the test.

The authors wish to express their sincere appreciation to Dr. D. A. Sutherland, Dr. Seymour Eisenberg, Dr. Ben Friedman, and Dr. Henry Lanz for their interest and valuable assistance in the preparation of the manuscript.

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A SELECTIVE METHOD FOR THE ISOLATION OF LISTERIA MONOCYTOGENES FROM MIXED BACTERIAL POPULATIONS

MOLLIE E. MCBRIDE, PH.D.,* AND KENNETH F. GIRARD, PH.D.**
HALIFAX, NOVA SCOTIA

INTRODUCTION

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Previous attempts made by Gray[2] using tellurite and sodium azide and by Shimizu[3] using a nitrofuran compound called guanofuracin (5-nitro, 2-furfuryl-idene, aminoguanidine hydrochloride)† have been shown in our experiments to have shortcomings either by inhibition of *L. monocytogenes* growth or by lack of selectivity.

Our experiments have been directed toward the evaluation of various chemical and physical agents in the hope that one or more of these might, when incorporated into a satisfactory basal medium, make possible selective cultivation of *L. monocytogenes* from mixed cultures containing small known numbers of this bacterium.

MATERIALS AND METHODS

Cultures.—NTCT strains of *L. monocytogenes* numbers 7973, 5348, 5105, and 5245 were used representing, respectively, *L. monocytogenes* Paterson serotypes 1, 2, 3 and 4.[4] Other organisms employed for testing the selectivity of the medium were *M. pyogenes* var. *aureus*, *E. coli*, *Ps. aeruginosa*, *P. vulgaris*, *Str. faecalis*, *Streptococcus pyogenes*, and *Bacillus subtilis*.

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†Commercially available in Japan.

necessity of eliminating the leukocytes before injection became apparent when a 220 per cent error was made in a blood volume determination on a patient having a leukocyte count of 400,000 c.mm. (3) Hemolysis, although uncommon, may not be perceived unless centrifugation is employed.

The results of this study demonstrate that the procedure commonly employed to label red cells with Cr^{51} may be shortened without seriously compromising the accuracy of the method. The procedure, thus shortened, may be quickly performed under circumstances which are considered emergent, yet at the same time the accuracy of the original method may be retained substantially.

This method has been applied in pre- and postoperative blood and fluid replacement problems. The rapidity with which the blood volume may be determined by this method should widen its field of usefulness. The method accurately measures only the red cell mass; the plasma and total blood volumes are estimated from the venous hematocrit.

SUMMARY

The procedure for determining the blood volume in which radiochromium is used has been modified for the purpose of completing the determination in a minimum length of time without significantly compromising the accuracy of the test.

The authors wish to express their sincere appreciation to Dr. D. A. Sutherland, Dr. Seymour Eisenberg, Dr. Ben Friedman, and Dr. Henry Lanz for their interest and valuable assistance in the preparation of the manuscript.

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Media.—For initial cultivation of cultures in preparation for the testing in different media, heart infusion broth (Difco) was used.

Various types of prepared media were tried for a basal medium in testing various experimental agents. In the preliminary stages, tryptose agar (Difco) was used, and as experimentation progressed, glycine agar base* was tried, and finally phenyl ethanol agar (Difco) was employed as a basal solid medium, and tryptose phosphate broth (Difco) as a basal enrichment broth. Tryptose phosphate agar was used as a control throughout.

The basal medium was rehydrated and autoclaved at 15 pounds pressure for 15 minutes, then cooled to 50° C. The experimental agents tested were dissolved in distilled water to suitable concentrations, sterilized by filtration, and added to the basal medium, aseptically, in the concentration required. The media were poured into sterile Petri dishes then placed in an inverted position at 37° C. to dry overnight.

Quantitative Testing Methods.—An overnight broth culture of each of the strains of *L. monocytogenes*, *E. coli*, and *M. pyogenes* was used as inoculum for the experimental solid media using the drop plate method,[5] with 6 drops per plate. Colony counts were done following incubation at 37° C. for 48 hours. The mean of 6 counts was taken for each culture grown on each of the experimental media and compared with the mean obtained for cultures grown on control medium, the results being expressed in percentage inhibition of growth.

Qualitative Testing Method.—When quantitative experiments indicated that a particular agent might be useful for selection, work was initiated with mixed cultures. Tryptose phosphate broth was incubated with one standard loopful (10^{-3} ml.) of a 12-hour broth culture of the following: *M. pyogenes* var. *aureus*, *E. coli*, *Ps. aeruginosa*, *Proteus vulgaris*, *Str. faecalis*, *Str. pyogenes*, *B. subtilis*, and *L. monocytogenes*. Experiments were run in quadruplicate, each with a different *L. monocytogenes* strain. The mixed culture was incubated at 37° C. for 36 to 48 hours, then streaked on the surface of agar containing the selective agent, and incubated for 48 hours at 30° C. The isolation of numerous colonies was considered to be a good result.

RESULTS

Substances that were tried individually and in various concentrations, but found to be of little value, include the following: *sodium chloride, glucose, changes in pH, potassium tellurite, guanidine hydrochloride, aminoguanidine, benzyl chloride, ethyl alcohol, phenobarbital sodium, boric acid, sodium thiosulfate, and lysozyme.*† Media containing rhamnose as the only source of carbohydrate in the presence of the indicator, phenol red, also did not prove useful.

Substances found to be of some value were guanofuracin, Furacin‡ (5-nitro, 2-furaldehyde semicarbazone), and Furadantin‡ [N(5-nitro-2-furfurilidene)-1-amino hydantoin.] Results are shown in Fig. 1. The concentrations of these 3 nitrofuran compounds were those predetermined by viable counts to offer maximal growth of *L. monocytogenes* strain 7973, Serotype 1. Note that at these concentrations, little or no growth of either *E. coli*, or *M. pyogenes* occurs. On the other hand Furadantin shows a distinct inhibitor effect on *L. monocytogenes* strain 5241, the Serotype 4 organism, less on the Serotype 3, and none on the Serotype 1 and 2 organisms. Both guanofuracin and Furacin show excellent selection with little or no *L. monocytogenes* inhibition in the concentration employed. Our results with guanofuracin agree essentially with Shimizu's except that we found it necessary to employ a final concentration of 1:50,000

*From the Case Laboratories, Chicago, Ill.
†National Biochemical Co.
‡Eaton Laboratories, Norwich, N. Y.

rather than the 1:10,000 used by him in order to obviate growth inhibition of one or more of the test *L. monocytogenes* strains. These results also indicate that Furacin at 1:100,000 final concentration may be substituted for guanofuracin. In addition to the above, several commercially available prepared selective media were tried for use as basal media. Glycine Agar and Glycine Tellurite were examined quantitatively in this respect and the results appear in Table I.

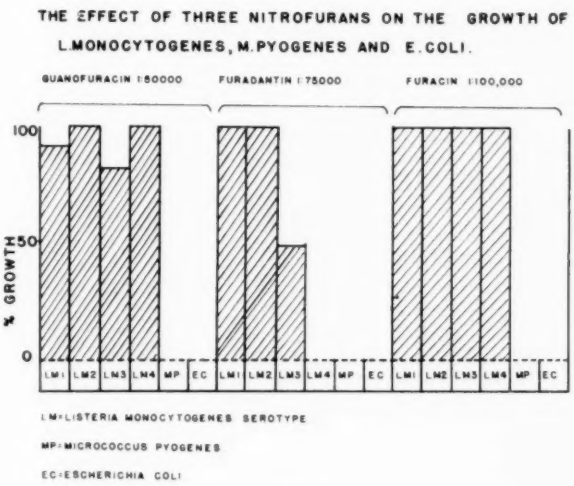

Fig. 1.

It can be seen that glycine tellurite produces considerable inhibition of all the strains of *L. monocytogenes* tested. Glycine agar base, however, showed inhibition to *Str. faecalis* and *E. coli*, but very little for *L. monocytogenes*.

Phenyl ethanol agar which is used as inhibitor of gram negative organisms in general and Proteus swarming in particular, was also examined. *L. monocytogenes* cultured on this medium, showed no inhibition of numbers or colony size when blood was added.

As a result of the observations and experiments described above, the following method is recommended for the isolation of *L. monocytogenes* from mixed bacterial populations:

Enrichment Broth.—Tryptose phosphate broth (Difco) rehydrated and autoclaved at 15 pounds pressure for 15 minutes with added Furacin 1:10,000 to make a final concentration of 1:100,000.

Selective Medium.—Phenyl ethanol agar base (Difco) containing tryptose 1 per cent, Bacto extract 0.3 per cent, sodium chloride 0.5 per cent, Bacto agar 1.5 per cent and phenyl ethanol 0.25 per cent. To this is added lithium chloride 0.05 per cent, glycine 1 per cent, and blood 5 per cent.

Procedure.—The specimen to be cultured is inoculated into the enrichment broth and incubated at 37° C. for 48 hours. Subinoculation is then made by

streaking on to the selective solid medium and incubating again for 24 to 48 hours at 37° C.

Using the above method *L. monocytogenes* could be isolated quite readily from mixed cultures containing *M. pyogenes* var. *aureus*, *E. coli*, *Ps. aeruginosa*, *P. vulgaris*, *Str. faecalis*, *Str. pyogenes*, and *B. subtilis*. *L. monocytogenes* produced gray colonies showing no zone of hemolysis. Other microorganisms present following this procedure (but considerably inhibited in numbers) are *B. subtilis* and *Str. faecalis* whose cultural characteristics permit easy separation from those of *L. monocytogenes*.

TABLE I. EFFECTS OF DIFFERENT MEDIA ON GROWTH

ORGANISM	PER CENT INHIBITION	
	GLYCINE TELLURITE	GLYCINE AGAR BASE
L. monocytogenes 1	100	0
L. monocytogenes 2	100	0
L. monocytogenes 3	100	25
L. monocytogenes 4	100	18
M. pyogenes	0	0
Str. faecalis	100	100
E. coli	100	100

DISCUSSION

This work has concentrated on finding a medium suitable for the rapid isolation of *L. monocytogenes* from mixed cultures. The medium and method described above have been found to be useful in separating *L. monocytogenes* from experimental mixed cultures. This method however, has not been examined extensively with clinical materials. There is still reason to believe that some specimens, particularly tissue, require refrigeration in enrichment broth prior to plating, as advocated by Gray,[6] in order to obtain maximum sensitivity. These difficulties are presently being investigated and have been reported on by Bearns and Girard.[7]

A prime difficulty encountered in this work was the physiologic differences discovered in the 4 serotypes of *L. monocytogenes*, which presumably hamper the isolation of the organism from clinical materials as well. This aspect was not pursued at this time, but it is felt that this may be a very fertile field for future investigation.

SUMMARY

A method and medium have been developed for the isolation of *L. monocytogenes* from mixed cultures as it commonly occurs in biologic materials. This consists of an enrichment broth in which the organisms are incubated for 18 hours, then streaked on a solid selective medium. *L. monocytogenes* produces gray colonies in 24 hours. Other organisms present could be easily differentiated by their appearance.

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ELIMINATION OF BACKGROUND STAINING IN THE PERIODIC ACID–SCHIFF METHOD FOR PROTEIN-BOUND CARBOHYDRATES IN PAPER ELECTROPHORESIS

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IN SPITE of the fact that paper electrophoresis of serum protein has become a routine procedure in many clinical laboratories,[1] methods for paper electrophoresis of protein-bound carbohydrates (PBC) have not been similarly perfected. The analysis of PBC in serum has proved to be useful in the clinical study of many conditions, including neoplastic diseases,[2] collagen disease, especially rheumatoid arthritis,[3, 9] blood dyscrasias,[4] and tuberculosis.[5] The procedure most frequently employed is that of Köiw and Grönwall[6] who have adapted the Hotchkiss[7] periodic acid–Schiff reaction to paper electrophoresis. Björnesjo[8] and Laurell[9] have also described methods for using the periodic acid–Schiff reaction for PBC. One of the major shortcomings of this method is the variable background staining of the paper strips, thus making exact quantitation of the periodic acid–Schiff reaction difficult. In this investigation we have made a systematic study of some of the factors which influence intensity of background, uniformity of staining, and reproducibility of results in the periodic acid–Schiff reaction for protein-bound carbohydrates. The solutions have been standardized and a simplified procedure was evolved.

PRELIMINARY OBSERVATIONS ON METHOD

To eliminate the background staining in the Köiw and Grönwall[6] method, the following modifications were found to be significant: (1) elimination of ethyl alcohol from all reagents, (2) reduction of oxidation time in periodic acid solution, (3) reduction of the cupling time with the fuchsin reagent, and (4) standardization of methods for preparing all reagents, particularly the fuchsin solution.

In Fig. 1 the influence of duration of periodic acid oxidation upon intensity of background is shown. After 1 to 2 minutes of oxidation hardly any discernible background could be observed on the paper strips. However, upon pro-

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longation of the periodic acid oxidation a progressively darker background was observed. This is most likely caused by oxidation of cellulose by periodic acid with formation of carbonyl groups.[10] A similar progressive intensification of background was obtained by increasing reaction time in the fuchsin sulfite solution.

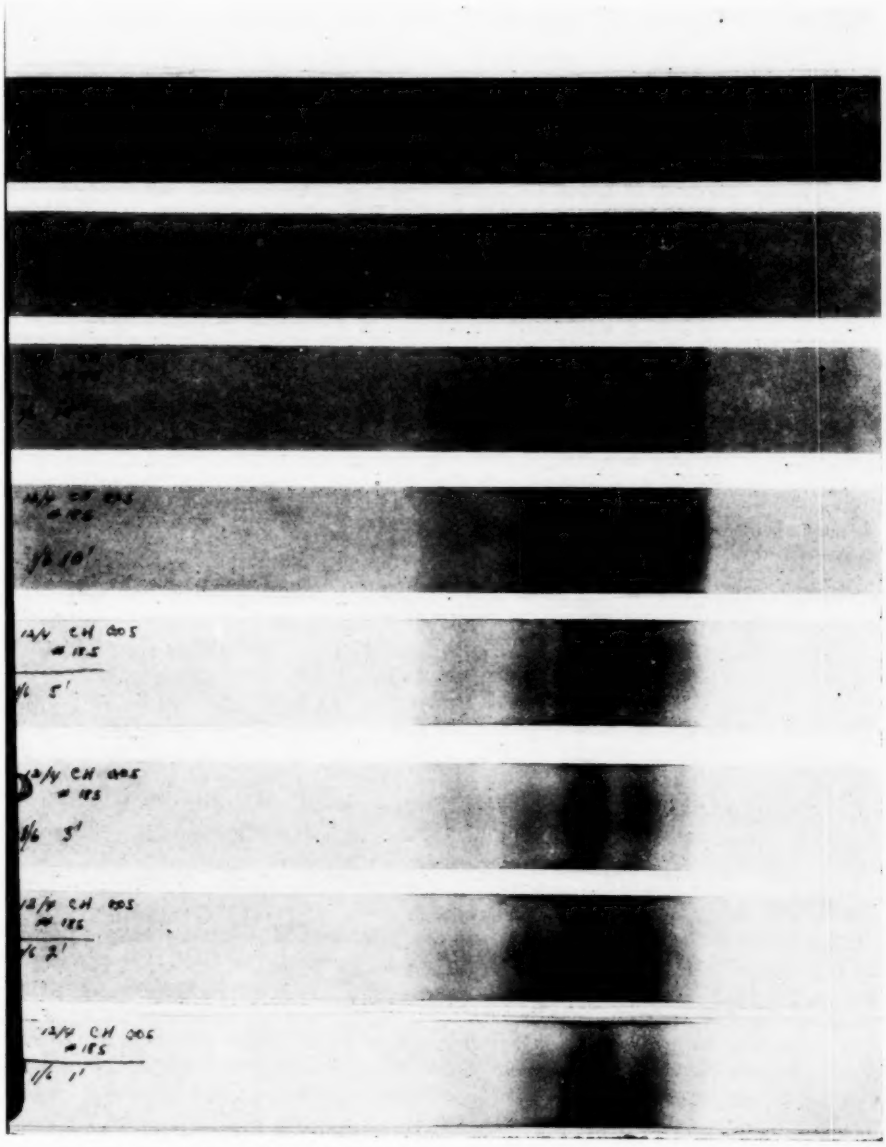

Fig. 1.—Effect of periodic acid oxidation on extent of background color. Strips allowed to oxidize in 1 per cent periodic solution from 1 minute to 30 minutes, and then the rest of the periodic acid–Schiff reaction was completed. Reading up: (1) 1 minute, (2) 2 minutes, (3) 3 minutes, (4) 5 minutes, (5) 10 minutes, (6) 15 minutes, (7) 20 minutes, and (8) 30 minutes.

Several other factors contributed to background staining. The use of rinses between reagents was found to be unnecessary when the strips were allowed to drain before being placed in the next reagent. The presence of small quantities of aldehyde in most samples of ethyl alcohol was probably responsible for some background staining. The method for preparing the fuchsin solution was found to be critical, especially with respect to rapidity of color development as well as shade of the bands.

MATERIALS AND METHODS

Periodic Acid.—Dissolve 10 Gm. periodic acid (H_5IO_6)* and 0.2 Gm. sodium acetate ($NaC_2H_3O_2 3H_2O$)† in 1 L. water. This reagent may be reused once or twice. It is stable for 2 weeks. It must be kept in the dark.

Reducing Solution.—1. Solution A: 50 grams potassium iodide (KI)†: 50 grams sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$)† dissolved in 1 L. distilled water.

2. Solution B: Thirty-five milliliters 1 N HCl diluted to 1 liter with distilled water.

3. Mix A and B just before use in proportion of 2 parts A to 3 parts B (400 ml. to 600 ml.).

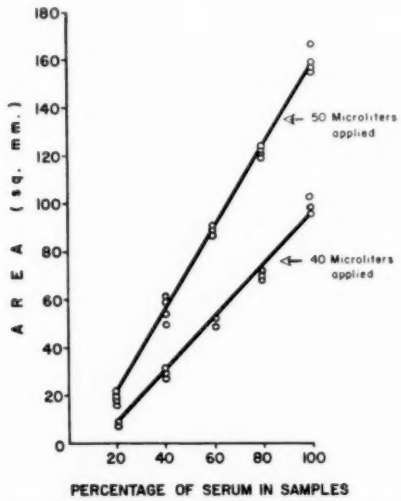

Fig. 2.—Relationship between intensity of color and amount of serum.

Schiff Reagent.—Dissolve 10 grams basic fuchsin‡ in 1 L. of distilled water heated to 90 to 95° C. Allow to cool below 40° C., add 20 grams potassium metabisulfite ($K_2S_2O_5$),† shake, add 200 ml. 1 N HCl, shake, and allow to stand overnight in the dark at 4° C. The next morning add 3 grams decolorizing carbon,§ and filter by suction through a Buchner funnel. The Buchner funnel is prepared by pouring an aqueous suspension of Johns Manville Celite Analytic Filter Aid over Whatman No. 1 filter paper while under suction. The solution of the Schiff reagent should be crystal clear and colorless. It should be kept cold in a dark bottle when not in use. The solution should not be kept for more than a month. It should be discarded as soon as it turns slightly pink.

*G. Frederick Smith Chemical Co., Columbus, Ohio.

†Baker Analyzed Reagent, J. T. Baker Chemical Co., Phillipsburg, N. J.

‡Biological Stain Commission, H. J. Conn, Geneva, N. Y., Certification No. NF 66, C.I. No. 677, 90 per cent dye content, National Aniline Division, Allied Chemical and Dye Corp., N. Y., Cat. No. 434.

§Norit A, Pfanstiehl Chemical Co., Waukegan, Ill.

Sulfite Rinse.—To 1 L. of distilled water add 8 grams potassium metabisulfite. Shake and add 20 ml. concentrated HCl. This solution should be used within a few days.

Electrophoresis.—Fresh serum was applied to Whatman 3 mm. strips (thick paper), using the Spinco Model R apparatus and the Spinco procedure as described in Spinco manual RIM-5.* Fifty microliters of serum was applied to each strip. Separation of the serum protein was achieved with a current of 5 Ma., over a period of 16 hours, using a cell containing 8 strips in Veronal buffer (0.075M, pH 8.6).

Staining Procedure.—†1. Two minutes in periodic acid. (Drain off excess solution after each step, by tilting rack.)

2. One minute in reducing solution. Repeat. (Strips must be white before proceeding to the next step.)

3. Leave in Schiff reagent for 2 to 3 minutes, but not more than 5 minutes.

4. (Optional) If background looks highly colored at this point, wash out part of the excess fuchsin by immersing rack in running tap water, tilting the container, for a period of 3 minutes.

5. Rinse in bisulfite solution for 2 minutes and repeat rinse in new solution for 4 more minutes.

6. Blot strips between 2 layers of clean blotting paper.

7. Dry in forced draft oven on Spinco drying rack at 37° C. for 10 to 15 minutes.

8. Scan strips immediately with Spinco Analytrol Model RB* using a 550 filter in the front and rear filter holders, the B-5 cam and the 4 mm. slit. If the 1½ mm. slit is used, a neutral density filter either 0.3 or 0.6 must be inserted in front of the rear filter holder.

RESULTS

Relationship of Quantity of Serum to Intensity of Color.—In order to evaluate the method with respect to deviation from Beer's Law, varying amounts of the serum were applied to paper and allowed to migrate for 2 hours in the electrophoresis cell, after which the strips were stained. A constant volume of fluid, 40 or 50 μl, was applied to the strips. The dilutions varied from 20 per cent to undiluted serum. The areas for each quantity of serum were determined by the Analytrol. The results are shown in Fig. 2.

It will be observed that a straight line relationship was obtained between 20 per cent and undiluted serum, using either 40 or 50 μl fluid. The results show that under the conditions described there was no appreciable deviation from Beer's Law.

Error of Method.—The standard deviation of the method was tested by doing multiple determinations on 3 different sera similar to the procedure reported by Björnesjo.[8] These results are shown in Table I. The standard deviations of the various fractions in each serum differed, indicating that under the conditions of separation employed in these experiments each serum appears to show its own set of characteristics.

Normal Values.—Sera from 48 healthy adult blood donors, both men and women, were tested. The sera were kept in the refrigerator at 4° C. and no serum older than 3 days was tested. The arithmetic means, standard deviations, and ranges are summarized in Table II.

*Spinco Division, Beckman Instruments, Palo Alto, Calif.

†Liter quantities of reagents were used along with the Spinco stainless steel staining rack and plastic trays.

TABLE I. ERROR OF METHOD IN DETERMINING THE RELATIVE DISTRIBUTION OF PROTEIN-BOUND CARBOHYDRATES

SERUM	NUMBER OF DETERMINATIONS	STANDARD DEVIATION OF MEAN*				
		ALBUMIN	ALPHA$_1$	ALPHA$_2$	BETA	GAMMA
A	36	1.7 (10.2)†	1.4 (17.9)	2.0 (27.1)	2.0 (30.8)	1.9 (14.0)
B	48	3.3 (14.7)	1.9 (13.0)	2.2 (21.9)	2.9 (29.3)	3.5 (21.1)
C	40	3.3 (11.5)	4.6 (20.4)	3.0 (24.4)	5.1 (29.6)	4.5 (14.1)

Fifty microliter aliquots of the same serum applied to Whatman 3 mm. paper strips, stained by the simplified method after electrophoresis.

* $\sigma = \sqrt{\frac{\Sigma d^2}{N-1}}$

σ = standard deviation.
d = deviation from the mean.
N = number of specimens.
†Arithmetic averages in parentheses.

Stability of Strips.—The stained strips can be kept at –20° C. for more than 6 months without intensification of the background. At room temperatures a progressive increase in background was observed.

DISCUSSION

In the modified periodic acid–Schiff method described, the control of background is achieved by elimination of extraneous sources of aldehyde. The periodic acid–Schiff procedure on paper strips apparently does not require the use of alcohol in any of the reagents. Oxidation of the strips for over 5 minutes in periodic acid gives irreversible background from the carbonyl groups generated by the oxidation of cellulose[10] (Fig. 1). The reaction time in fuchsin was accelerated by increasing its concentration from 0.1 per cent to 1 per cent, thus making it possible to obtain rapid staining of the applied material without allowing weakly reacting carbonyl groups in the paper itself to combine with the fuchsin reagent.

In order to obtain a readable level of color, it is necessary to separate at least 50 μl serum. Apparently poor electrophoretic separation is obtained by employing large quantities of serum. In the protein method,[1] where much greater precision of measurement is obtained, either 6 or 10 μl serum are separated, depending on the thickness of the paper. It is also possible that periodic

TABLE II. NORMAL VALUES OF PBC OBTAINED FROM 48 ADULT BLOOD DONORS

FRACTION	ARITHMETIC MEAN	STANDARD DEVIATION	RANGE
Albumin	11.0	3.5	7 to 15
Alpha$_1$	18.1	3.5	14 to 22
Alpha$_2$	24.8	5.4	19 to 31
Beta	29.4	5.4	24 to 35
Gamma	16.7	4.1	12 to 21

Fifty microliter aliquots of each serum applied to Whatman 3 mm. paper strips, stained by the simplified method after electrophoresis.

acid–Schiff reactive material in serum other than PBC occurs. This material may not migrate in any predictable fashion, thus blurring the boundaries of the major PBC components.

SUMMARY

A modified periodic acid–Schiff method is described for the determination of protein-bound carbohydrate in serum separated by paper electrophoresis. A major disadvantage of previous methods was formation of a variable background which interferes with photometric scanning of the stained paper strips. Uniformly colorless backgrounds were produced in our method by: (1) elimination of alcohol from the reagents, (2) minimum oxidation of the paper strips in periodic acid, (3) minimum coupling time in fuchsin sulfite, and (4) standardization of the methods for preparing all reagents. A straight-line relationship was observed between the amount of color developed and the quantity of serum. The error of the method was determined and ranged from 1.4 per cent to 5.1 per cent, depending on the fraction of serum. Normal values (per cent total protein-bound carbohydrates) for adults were determined, and these were albumin 11.0 ± 3.5, alpha$_1$ 18.1 ± 3.5, alpha$_2$ 24.8 ± 5.4, beta 29.4 ± 5.4, and gamma 16.7 ± 4.1.

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THE HAZARD OF ORALLY PIPETTING TRITIUM OXIDE

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THE obvious hazards involved in pipetting radioactive solutions can be attributed to two causes: (1) A pipetting accident caused by overzealous aspiration and (2) the inspiration of vapor. These two possible hazards can be controlled by the simple issuance of a decree forbidding the oral pipetting of radioactive materials. Nevertheless, with the increasing availability and use of tritium compounds of high specific activity, for example, water and compounds which give rise to water by their metabolism, it seemed of interest to obtain an estimate of the quantity of vapor inspired during the act of pipetting.

For these determinations a simulated oral pipetter consisting of a greased hypodermic syringe fastened by rubber tubing to the pipette being tested was used. The water vapor entering the rubber tubing and syringe was equilibrated with a known volume of water and an aliquot assayed for tritium.

As will be seen in Table I, the consequence of orally pipetting tritium oxide is the aspiration of about 5 to 70 mμl. of water per operation. When one considers the number of such operations which are frequently carried out in the laboratory and that water-H^3 of a specific activity of 1 μC per mμl. (which is only 0.03 per cent T_2O; 100 per cent T_2O has a specific activity of 3×10^9 μC per ml.) is in common use, it becomes apparent that with each pipetting operation one's isotopic enrichment may become appreciably increased.

TABLE I. ASPIRATION OF WATER VAPOR DURING PIPETTING

PIPETTE	SIZE ML.	MILLIMICROLITERS OF WATER ASPIRATED TRIAL			
		1	2	3	4
Long-tip	0.1	0.3	5.6	4.6	4.8
Long-tip	0.2	0.1	8.4	—	—
Long-tip	0.5	0.4	5.7	11.0	5.3
Volumetric	5.0	3.0	61.0	73.0	67.0

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